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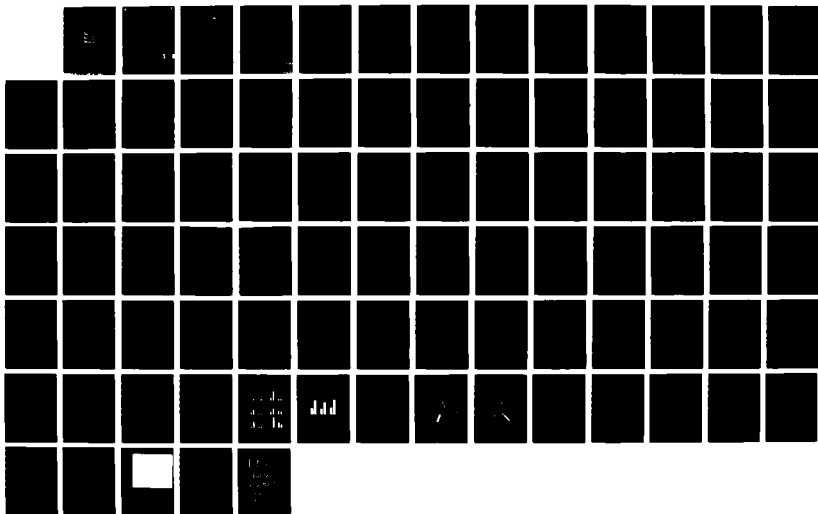
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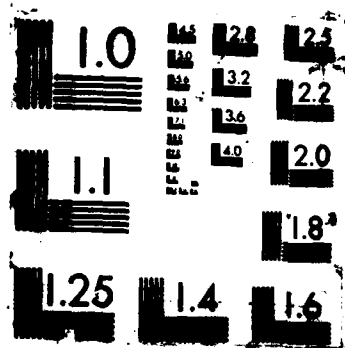
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THREE-DIMENSIONAL COMPUTER GRAPHICS  
BRAIN-MAPPING PROJECT

Annual Report

Robert B. Livingston, M.D.

March 15, 1987

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## FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the procedure or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council (NIH Publication No. 86-23 Revised 1985).

## SUMMARY

Progress during the first twelve months has come a long way toward developing and improving "brain mapping technology/methodology" (SOW 1). An advanced operating system has been established. Demonstrations of brain mapping in three species (SOW 2, 3 & 4) are in progress, and a number of procedural trials have been evaluated. During the course of completion of initial efforts at computer-assisted brain atlas development, the system can be greatly improved by expanding hardware and software for speedier management of large data representations and for user-efficient image editing capabilities. Ultimately, three dimensional memory storage, image rendering, display and analysis, and inference-guided, automatic mapping of neuroanatomical features will be necessary. Delays in the delivery of state-of-the-art equipment and software from vendors were the principal factors that retarded anticipated progress.

The Brain Mapping Project involves two parallel endeavors:

I) Development of a primitive brain mapping system from commercially available components; II) Determination of the feasibility of the system by testing it in the course of mapping brains of rat, monkey, and human

Four general objectives were identified in the Scope of Work:

1. Develop and Improve Brain Mapping Technology/Methodology
2. Acquire and Create Morphometrically Correct Large Scale Maps
3. Acquire and Create Selected Sub-Structure Maps
4. Create Cytoarchitectural and Myeloarchitectural Reference Brain Atlases

Productivity during the year is described in regard to each of these objectives, along with descriptions of progress toward subordinate objectives as further specified in the Scope of Work.

### 1.0 Develop and Improve Brain Mapping Technology/ Methodology.

The goal of this Section is to devise a brain mapping approach and to select, acquire and implement hardware appropriate for computer-assisted brain mapping. The objectives are to improve the accuracy and speed of digital image acquisition and analysis.

Data generated by these improved methods are to be used to produce computer graphics (synthetic) maps of whole



tissue sections and to create three dimensional "atlases" out of reconstructed images that include the boundaries of major internal brain structures as well as external and ventricular surfaces.

The hardware-software techniques introduced this year have yielded objective gray level thresholding of boundaries for outlining gray levels encountered in registration control images. Such page-graphic contours of structures are being assembled for surface renderings, using multiple wireframe images, creating surfaces by solid renderings (ContourView-Solid) and by using polygons for surface shading (i.e., Movie.BYU).

#### **Protocols Designed to Achieve the Brain Mapping Methodological Objectives:**

The design makes use of two types of images to generate aligned sections for spatially accurate maps and for three-dimensional "atlas" reconstructions. The protocol involves several steps: 1) acquisition of images from the sectional blockface of the brain after each knife pass (Registration Control Images, RCIs); 2) utilization of each RCI as a standard for computerized correction of distortions and alignment of the corresponding Tissue Section Images (TSIs); 3) analysis of the RCIs and TSIs by digitally contouring the external surfaces, ventricles, and major internal structures obtainable from tissue sections; 4) higher resolution analysis of TSI's to determine x, y, and z coordinates for structural boundaries of loci of identified neurons or neuronal densities from mosaics of microscopic fields; 5) production of three dimensionally reconstructed computer graphics "atlases" of tissue sections; and, 6) computer graphic reconstructions of surfaces defined by contours from serial sections, defining major internal structures. Data from RCIs and TSIs are used to label and to catalog structures for identification in page maps and surface renderings. RCIs and the de-deformed TSIs are three-dimensionally accurate and provide templates for the development of "atlases".

#### **Hardware and Software Implementations to Improve the Speed and Accuracy of Brain Mapping and Histological (Neuronal Localization and Tissue Density) Data Acquisition (SOW 1.1 & 1.2; 3.1 & 3.2)**

A brain mapping protocol using commercially available technology was implemented as rapidly as feasible. To maintain the facility for spanning magnifications from gross brain morphology to microscopic tissue image scales and to satisfy the goal of three-dimensionally accurate serial section reconstructions using computer graphics, we acquired the most powerful high resolution data acquisition systems available within our budget (MegaVision); together with a reputable host processing system (MicroVax).

We established two such systems in order to be able, more rapidly, to acquire, process and edit: 1) whole RCI brain surface and TSI macroscopic tissue section images for 3-D brain "atlas" reconstructions; 2) TSI microscopic data from which to create mosaic synthetic images that manifest neuronal distributions throughout whole brain sections; and, 3) "functional neuroanatomy labels" achieved by analysis of molecular specific stains for identifying and localizing major neurotransmitter systems and specific cell receptors for later "insertion" into 3-D whole brain reconstructions. Three independent computer controlled microscopes contribute data for brain mapping applications by means of this system.

Selection criteria for data acquisition hardware and software installations aimed for rapid acquisition of whole brain surface images from blockfaces as successive serial sections were removed. Microscopes and software for computer-assisted light microscopy serve to localize accurately in x, y, z coordinate space various types of specific neurotransmitters and specialized receptors. This requires the use of high resolution Nomarski and fluorescence microscope systems that provide additional capabilities (beyond the TSI microscope) for mosaicing from large tissue sections.

The combined image processor and host computer are interfaced to the LKB cryomicrotome and to a back lighted-macroscopic section imaging system. Microscopes with motorized stages and cameras were ordered but the stages did not arrive in time for interfacing this year. Specialized software was written and implemented to reinforce software packages from vendors for control of these systems and for image analysis. Reliable but slow acquisition of images and digital analysis of the brain surface and structural distinctions between white and gray matter were achieved using the macroscopic imaging of the system during the first year.

Image analysis was obtained by a cascade of image processing routines that culminate in detection of the outside edges of the specimen, the ventricular surfaces, and a few major internal structures. These operations include corrections for unevenness of the field of illumination, image normalization to the full 256 gray scale levels, thresholding to contour the outside of the specimen, thinning of the boundary to a single pixel line, and edge following that defines contours that will be stored. These contours are connected vectors that enclose an object.

Contouring results in mapping the outside and ventricular surfaces of the brain and some major internal structures. Three dimensionally defined data belonging to internal structures are segmented into defined objects which have labels and are displayed as page maps using the laser printer or plotter.

Contours can also be segmented to make up individual structures. By retaining their alignment and correct 3-D interrelations with other structures, they can be gleaned from individual and collective three dimensional reconstructions. Four modes of reconstruction are available: wireframes using the Digit system, CellView of Brainmate, or Movie.BYU. Solids rendering is projected by Contour.View and Movie.BYU. The Contour.View package of software fills the contours and stacks the sections as "voxel boxes." The wire frame-net approach generates polygons by triangulation between contours. The polygons are displayed as a wire frame or as a rendered surface using controllable lighting characteristics.

#### **Solutions Implemented for Improvement of X-Y-Z Alignment and Prevention of Distortions. (SOW 1.3 & 1.4)**

Deformations affecting the thin tissue sections are determined by comparing the undistorted RCI with the TSI. The RCI contains some additional information depending on translucency of the tissue and mounting media. Internal biological fiducials are abundant in nearly every section. We have used red latex in some preparations and India ink in others to make blood vessels stand out. De-deformation starts by approximating the TSI to the RCI using x, y translate and rotate features of the MegaVision. Rotation has not yet been supplied by MegaVision and is scheduled for installation in mid-June. The supposed twelve differential warping points provided by MegaVision do not do more than four or five points at best; beyond that number the machine stalls. We are greatly in need of more powerful algorithms for 2-D warping, and for, as yet unprecedented, 3-D warping.

#### **2.0 Acquire and Create Morphometrically Correct Large Scale Brain Maps**

The objective is to explore brain mapping capabilities by generating limited brain maps of rat and monkey, and a preliminary "sketch" map of the human brain. Brain mapping protocols utilizing combinations of RCIs and TSIs of rat brains have been used to explore, test and optimize brain mapping procedures.

In addition, the rat was used to test various methods for brain stabilization by fixation to minimize distortions (i.e. shrinkage or swelling), maximum contrast differentiation, orientation, alignment for microtomy, and image frame acquisition from the blockface and from tissue sections. Other procedures were tested on monkey and human brains for preparation of intact specimens for sectioning and for analysis of one megabyte RCI and macroscopic TSI images.

Results show that the present hardware-software approach is reasonably effective for acquiring images from

the blockface and from tissue sections. The CCD camera was disappointing, as it lacked responsiveness to a good deal of the range of colors customarily used for neuroanatomical histological staining. Nevertheless, the 1024 x 1024 video scanner provided by MegaVision was acceptable for image acquisition. These images provided boundaries for the brain and ventricular surface and the white matter masses and bundles. For macroscopic analysis of TSI's, the MegaVision can define these structures and densely stained cell groups. These frameworks provide a lattice on which subregions can be defined and used to locate structures, labeled neurons from specialized staining techniques (antibody tagging with fluorescent dyes, special enzymes, etc.) and cytoarchitecture of various regions of the neuropile.

A number of rats have been prepared through the premicrotomy preparatory stages to refine procedures required to produce accurate records using digital frame analysis. Three of these specimens have been sectioned and the blockface images recorded as a frame for sectioning at 200  $\mu$ m intervals. One of these is being analyzed for boundaries of the edges and internal structures. Final sets of rat brains were prepared and are ready to section for combined RCI and TSI image analysis.

Three monkey brains have been analyzed from previously prepared celloidin embedded serial sections, alternately stained for cells (Nissl) and fibers (modified Weil), and sliced precisely at right angles to one another in the three classical orthogonal planes. Because these Macaca mulatta specimens were carefully matched, female adults of the same body weight, three dimensional reconstruction permits each brain to serve as the template for the proper alignment of serial sections in the other two. These preparations have been useful for testing computer aided image extraction from stained preparations.

A number of human brains have been prepared for sectioning. Procedures for handling human brains for pre sectioning CT and MR scanning have been worked out; these include mounting these large specimens on the microtome. Other efforts using human material have been applied to existing whole human brain sections and MRI scans. Here, both interactive and automated boundary extraction have been tested. The large size of the human brain results in lower resolutions being generated on single frames. The mosaic approach will allow smaller overlapping fields to document TSI details.

### **3.0 Acquire & Create Selected Sub-Structure Brain Maps**

The goal is to map neuromarkers for acetylcholine, substance P, and a catecholamine in the rat, two-cell labels in

the monkey neocortex, cytoarchitecture of the human thalamus, and location of specifically labeled amacrine and ganglion cells of the retina.

Immunological labeling in the rat for acetylcholine neurons, substance P, and a catecholamine have been produced on serial sections as control preparations for subsequent studies. Acetylcholine and substance P labels have been mapped onto accurate outlines of brain sections. These data await generation of the digital rat atlas.

Analysis of various areas of the cerebral cortex for the density of specific types of neurons in localized regions is about 40% complete but the insertion of these data into three-space defined reference atlases awaits generation of a regional defined monkey brain wireframe "atlas" that is correct three dimensionally by being based on RCIs.

A map of the structural organization of the human thalamus has been created from the brain, SP-18, of the Yakovlev Collection. A 3-D reconstruction was obtained of 43 identifiable components of the thalamus taken from those brain sections. The nuclear aggregates composing the thalamus were outlined by interactive digital means at (0.7 mm). These were displayed as page maps using graphic plotting. The Evans and Sutherland vector graphics system was used to digitize the outlines of the whole thalamus together with outlines of each nucleus and subdivisions. Registration of the serial sections was achieved by reference to internal stereotaxic markers which were introduced before the brains were prepared for slicing. Composite wire frame computer images have been created to depict the human thalamus.

Additionally, the outlined nuclear regions were sampled at various magnifications for demonstrating the cellular types and cytoarchitectural organization. These were archived as references for each region as analogue images on an optical disc which is being indexed with accepted nomenclature for correlation to the 3-D graphic images.

Maps of ganglion cells and amacrine cells in the retina based on immunological labels have been defined for the rat and pigeon retinae. The retina represents a very important, highly localized circuit in which mapping of structure function relations can be especially explicit and informative. The location of specifically labeled cells for choline acetyltransferase and nicotinic acetylcholine receptors of amacrine and displaced amacrine cells across the retina show that the distributions are highly organized. This digital information will be formatted such that correspondence maps can be made once the microscopic stage is operative, and the high resolution recording procedures have been completed.

#### 4.0 Create cytoarchitectural and myeloarchitectural reference brain atlases

The first objective is to provide cytoarchitectural illustrations of human brain material obtained from the Yakovlev Collection, using Nissl and modified Weil stained preparations. Frame reference maps were made on an analogue disk. These have been referenced for access by acceptable nomenclature to facilitate reconstruction of the regional boundaries.

The second objective involves digitally rendered representations of external and ventricular surfaces and the white and grey matter surfaces of rat and monkey brains. For rat and monkey brains, we have been using Contour.View software from 3-D, Inc. for voxel rendered surfaces. This is now undergoing revision and we expect to have improved quality of representation, smoother surfacing, greater dimensions, all with more rapid evolution of images. Inclusion of Movie.BYU into the choices of our own system is planned and will give added dimensions in surface rendering for multiple objects.

## DETAILED ANNUAL PROGRESS REPORT

### Overview: Brain Mapping Tasks

The Brain Mapping Project seeks to achieve valid three dimensional mapping by taking advantage of the processes of physical sectioning to ensure correct alignment throughout the entire specimen. Each brain defines its own coordinate system as represented by its in situ dimensions. Information was obtained as contours and boundaries of internal structures and point loci of small objects in sequential sections. These graphic and primary records of frame grabbed images are stored in a Laboratory-Records Database for retrieval and incorporation into page maps and three-dimensional reconstructions.

The methods used to acquire these digital records involved hardware-software systems design consisting of commercially available hardware and the little commercially available software. The remainder of the software had to be developed in house in order to integrate the various components into a functional system.

In order to generate the contour records of internal and brain surface structure, two types of frame grabbed images were obtained. One was a Registration Control Image (RCI) which was acquired from the block face between sections. A second image is obtained as a whole-frame digitization of each tissue section prior to staining and mounting (MacroTSI), a third image is taken after staining and mounting (MicroTSI). The RCI image is used to define the general in situ Cartesian coordinates throughout the brain. MacroTSI images yield more detailed coordinates, which are then used, along with the RCI, to de-deform the MicroTSI image, and the subsequently corrected synthetic tissue image map.

Detailed information on internal brain structures was obtained from the TSI frames by recording at the microscopic level using a mosaic construction of the synthetic image. This combined RCI-TSI approach provides alignment and high resolution extraction of regional boundaries of features respectful of various stains and structural and chemical labels, and provides the framework for mapping detailed cellular information.

The recording of these three types of images requires hardware implementation and software that bring together computer technology, microtomy, microscopy, and database management. The design of this system must also implement

user-friendly menu controls for editing, recording, data keeping, analysis, and retrieval.

The principle used in the design plan is first to establish the system at a level of utility around equipment currently available on the commercial market and then to develop software and minor hardware modifications to achieve a new facility for brain mapping. This plan provides for further development around this functional system on which realistic tests of brain mapping can be made. The goal was to achieve as much automated boundary tracing as possible from frame digitized sections. A combined interactive and automatic boundary definition system had to be used to edit and record structures that could not be defined using a primitive gray-level imaging approach.

The plan was to begin with existing software components in order to achieve a working system for interactive software development. The transition from commercial products to a developmental system involved implementation of necessary software interfaces and drivers for peripheral operations. Now that a basic system has been established, modifications in graphic menus, sequences, image analysis, and database utility can be made to enhance and further facilitate the brain mapping operation. In some cases, entire aspects of software routines had to be re-written in order to optimize operations.

This section of the report describes the scientific and technical progress obtained thus far in generation of a brain mapping system and acquiring the requisite digitized anatomical data needed for the testing and creation of a computer-graphic brain map. There are two major aspects of this study: 1) Design and implement a hardware-software systems approach to brain mapping based on digital technology, and 2) Demonstrate the utility of the system on three species of brains by producing general "atlas" like representations of structures for 2-D and 3-D relationships. This brain mapping project consists of specifics that attempt to test the full range of possible endeavors required of brain mapping.

Research on a number of problems was carried out during the reporting year and included specimen preparation, histochemical staining, cryomicrotomy, video imaging and image pre-processing, virtual sectioning with computer tomography and magnetic resonance imaging, and the collection of tissue sections destined for microscopic analysis. We present, then, data from diverse experimental projects. At this current juncture the techniques used to generate these data are still prototypical, and may undergo varying degrees of refinement. The reporting is presented in four sections that follow the Scope of Work.



## SYNOPSIS OF WORK-SCOPE

- 1.0 Develop methods for acquisition of brain section images for mapping and reconstruction of surfaces.
- 1.1 Improve accuracy and speed of histological data acquisition
- 1.2 Improve registration and alignment for accurate registration of the 2-D and 3-D data histological sections
- 2.0 Acquire and Create Morphometrically Correct Large Scale Brain Maps
- 2.1 Prepare whole rat brain atlas of common structures using Tissue Section Images (TSI).
- 2.2 Prepare whole monkey brain atlas of common structures using Tissue Section Images (TSI)
- 2.3 Preliminary atlas of whole human brain
- 3.0 Acquire & Create Selected Sub-Structure Brain Maps
- 3.1 Neurochemical maps of whole rat brain based on neurotransmitter marker: acetylcholine, substance P, and a catecholamine
- 3.2 Neurochemical maps of monkey neocortex showing regional locations for two neurochemicals and illustrate with wireframe images
- 3.3 Prepare map of human thalamus based on Yakovlev collection
- 3.4 Map location of amacrine & ganglion cell based on structural-functional labels.
- 4.0 Create cytoarchitectural and myeloarchitectural reference brain atlases.
- 4.1 Human brain mapping of Nissl patterns of nuclei by example, stored on an optical disk.
- 4.2 Digital rendering of external and ventricular surfaces and the white and grey matter surfaces for rat and monkey brains.

## **1. Develop and Improve Brain Mapping Technology / Methodology**

### **1.0 Description:**

The design, acquisition and implementation of a Brain Mapping System was carried out to near completion in the first year. The objective was to improve the speed and accuracy of brain mapping by implementing a digital brain mapping approach. The system design was focused around the need for image acquisition from both the blockface for registration control images (RCI's) and mounted, stained brain slices, or sections, for tissue section images (TSI's). In addition, algorithms and user interfaces for image processing, and for external and internal contouring of brain structures in sections were created to define and display the surface and parts of the brain graphically. Additional specifications of the design were to achieve accurate spatial representations of brain sections so that the graphic maps and three-dimensional reconstructions of component parts of the brain would also be accurate. The product of the data generation is 2-D section-page maps in conjunction with wire frames of the brain surface and limited internal structures. This includes a capability for rendering of surfaces such as the gross brain structure, the ventricles and myelinated fiber tracts.

Because of the digital approach, hardware and software had to be assembled and a number of software routines written to integrate a brain mapping system. The design called for utilization of "off-the-shelf" hardware and as much software that could be practically used in order to achieve a test demonstration of a system within the time constraints of the contract.

The first section of the Scope of Work (SOW 1.0) is interpreted to mean that we will generate a hardware-software system from commercially-available system components that is effective for brain mapping. The development of the biological approach and applications are all considered under sections 2,3 & 4 of the SOW although they are an integral part of the SOW 1.0 objectives.

### **1.1 Hardware System Design and Selection:**

The objective is to design and implement a brain mapping system from commercially available components, test, compare and select equipment. The selection of components was made through tests and comparisons of equipment with the criteria of defining optimal image acquisition and analysis. (See Fig 1.0-2)

## Summary of Considerations for Design of the Brain Mapping System

1) Each brain should be recorded within its own Cartesian coordinate domain. The facility of digital transformations allow comparisons between brains at later times. Nevertheless, each species should have defined standard coordinate axes and sectioning should be maintained as closely as possible to a standard coordinate axis. This will maximize the effective data entry between brains for adding data from other sources to the primary record, i.e., standard digital atlas format can serve as a template for a digital brain framework.

2) Multiple types of data formats of tomographic slices must be collected and stored for database acquisition. The use of 2-D coordinates on serial slices is effective for generating a 3-D domain. Rasters containing optical densities for individual pixels compose one type of data format. Another type is points composing vectors which are unclosed. A second type is points representing central loci of defined objects. Transformation of 2-D slices to 3-D Cartesian coordinate data, irrespective of slices, can be generated later to reduce data and optimize the display and analysis.

3) Technical limitations in generation of voxel-boxel brains. A voxel/boxel brain record is desirable for both 3-space display and analysis. At this time such an approach is not deemed to be feasible because the amount of information that must be analyzed at any one time to achieve resolutions needed for brain mapping. Ideally, tomographic voxel records should be analyzed as a cubic voxel set on a cubic random access memory. The limitations on data accessing in 3-dimensional memory arrays is now only becoming technologically possible on small arrays. Important progress has already been made in the MRI field where boxel information is readily available. However, the data set is much smaller than that required in this brain mapping project.

The current project will utilize vector/point records of boundaries and point loci which will be carried through the 2-D and 3-D analysis and display procedures as well as being maintained as a ready reference for merging and compiling data from other sources. The analysis of vector and point (vector/point) information provides a considerable amount of data and also serves to reduce the amount of record data and information that must be carried through various stages of display, analysis and storage. With the current state of technology, it is felt that this is a practical means of generating brain maps.

4) RCI versus TSI images for restoration of the 2-D spatial domain for distortions and alignment. The procedure

defined by this method is to utilize frame grabbing of single-frame, whole-brain blockfaces as a Registration Control Image (RCI) and is used to realign sections and to warp images of tissue sections back to the coordinate domain of the blockface. Each preparation will employ structure-defined fiducial points as markers that are automatically determined or operator indicated for each of the two preparations. These are used in a combined warping and realignment procedure. The differential displacement among these local points will be defined by the amount of warp on the Tissue Section Image. In the future, it is hoped that automated aligning and warping of preparations will be possible.

5) Recording resolutions. The synopsis of information indicates a variable range of resolutions are needed to map overall brain dimensions and fine detail within sections. The RCI and boundary detection will be necessary to define location of identified cells and labeled regions within the nervous system. Furthermore, in order to define a precise location of boundaries, even at the global level, it appears that high resolution detail imaging will be necessary, particularly, if automated approaches are to be utilized.

In order for computer recognition to be employed, adequate digital information must be available in both 2-D space and in the optical density domain. The differentiation of boundaries between adjacent structures requires a combination of minimum spatial and optical density separations. It appears that each object must have a minimum spatial separation of 3 pixels, but, preferably 10 or more pixels are required to clearly achieve a spatial differentiation by most algorithms. The number of optical density levels in the image is a significant factor in object differentiation. In some images as few as 50 digital levels may be obtained for the entire image. Shifting and skewing the digitizing range is needed with high signal to noise differentials to maintain the full digital range (256) over the optical density range of the image.

6) Analysis of single frames of the blockface and tissue sections. Some information can be gained from the automated analysis of optical density on single 1024 squared frames. It will be possible to generate a surface outline of the brain, ventricles, and myeloarchitecture. Further detail will have to be gained by mosaic frame recordings through either using large array scanner (4000 line) or stage stepper devices which either move the camera or the preparation. Appropriate optical assistance will be necessary to provide relatively undistorted images of the field or else standardized warping will have to be applied in order that the mosaic images will be fit together.

7) Resolution in the Z axis determined by sections.

The resolution in the Z axis of sections will be much less than the X-Y axis because of the utilization of optical density for image generation and the thickness of sections. Thinner sections help to improve the resolution in 3-space, but generate less material for differential density production. This results in an increase in the signal to noise ratio.

A second factor, limiting this resolution, is due to falling off of the density of the object as its edge projects obliquely within the section. The result is much less mass occurring over parts of the object while a part maybe still entirely sectioned and thus maintains the maximum density level inherent in the object. As the object ends within the section and the trajectory follows a very oblique path through the mid-section level, the mass is greatly reduced. Therefore, the actual ending of the object over the two sections is uncertain.

Another condition exists in objects consisting of a thin walled rind. The transverse section of this wall in profile produces very sharp edges, however as the thin wall curves it projects as an oblique trajectory through the section, the density falls off rapidly with the angle that it passes. Thus, the back and front walls are virtually impossible to detect because of the translucency of the enface view. A partial solution to this problem may be represented by extrapolation of the boundary from other sections.

8) Resolution determined by optical density. One of the major limiting factors of automated image analysis has been in obtaining sufficient density levels whereby differentiations can be made between objects having nearly the same density. In the usual blockface preparation, the number of optical density levels may be less than 50 in 8 bit recording (256). Therefore, it is essential in future projects to extend the optical density levels range by increasing the bit level to 12 or 14. At the current time, 12 bits is possible, 14 bits remains questionable and 16 may only be achievable in future technological refinements. Even in this project, enhancement in density range values generated from section preparations could be of value to improve automatic methods. Generally, image processing is effective for averaging frame grabbed images, and subtraction of background. Some noise elimination algorithms can help but, except for maximum optical differentiation at the time of recording, none provide real gains in maximizing the number of optical density levels.

9) Data records for 3-D mapping. Three types of records should be kept: a) Primary data records of pixel/raster data of 8-bit optical density levels. This data is preferably in the 1024 squared or greater range in order to obtain spatial resolution of full brain sections.

The numerous overlapping images that are used to generate a high resolution record can not be stored at this time. b) Data files should represent the analyzed files consisting of points and vectors defining identified structures, cells, and regions of the brain. Each image file is referenced into an overall header file of each brain. Sufficient labeling of objects is necessary in order to maintain an accurate record of section mapping. A textual and numerical description composes the header file organized around a database format. These files provide a complete retrieval of information regarding the source of data, recording parameters, display and analysis parameters, definition labels, and attributes.

10) Analysis of data records of sections for two-dimensional parameters is essential for mapping information about numbers, densities, locations, shapes, sizes, orientations, and neighbor interrelationships for 2-space understanding. These values can be readily obtained from vector coordinates for boundaries and point loci records of individual sections through the brain.

11) Data analysis of 3-dimensional parameters related to 3-D brain maps. A number of parameters defined in the 2-D section analysis is applicable to 3-dimensional analysis of internal structures, shapes, sizes, orientation, spatial domain, and object neighbors. Such information should become a part of the database of brain maps and atlases. The presentation of this information should include a ready reference of the source and be easily related to the brain maps and 3-D atlas information.

12) Segmentation of images in three dimensions remains a major obstacle to reconstruction by interactive and automated methods. The reconstruction of objects in 3-space images from boundaries taken from serial sections poses numerous problems as to the identification of continuity between boundaries along the three dimensional axis. In the past, most reconstructions have dealt with non-prealigned preparations adding considerably to the difficulty. Most methods attempt reconstruction from stacked boundaries of surfaces by linking two adjacent sections. As a result, continuity of structure is impossible to define. This is because bifurcations and reentrant parts of the image can not be followed with such a limited amount of information. The continuity of these objects can only be detected by analysis of a large set of boundaries. The solution to having operator identification with labeling of boundaries is not entirely adequate because the operator has an inability to comprehend the full set of slices. A true three dimensional algorithm has yet to be defined. Such an algorithm is envisioned to search the internal surface of closed sets of boundaries for continuity throughout the set of slices. This approach, if it is entirely practical, however, will

require considerable computational time if high resolution is to be obtained.

13) A database construct needs to be developed in order to facilitate a generalized database structure whereby digital information can be accessed across a wide range of types and sources. Three types of databases are envisioned. The first is directly related to the project and is a Laboratory-Records Database. Considerations for the categories of data and the assigned attributes as well as a nomenclature scheme are a central issue to be dealt with in the data acquisition domain.

The second data structure is a Shared-Information Database which is the primary product of the research project. Although digital access is not a major requirement of this project, the digital presentation of data is essential for generating shared maps and atlases. The generation of digital maps and atlases provides a standard reference of defined brain regions and component locations which can be used by scientists to analyze their own data and as a format for contributing data to a common database. This requires transportability and standardization and is being considered in the design from acquisition stages to informational retrieval. Most important will be a hierarchy of graphic systems, that will allow degrees of facility for interaction with the same database.

A third database is envisioned to be a synoptic, synthesized base of information that can be addressed by interactive graphics. This has been termed a Shared-Knowledge Database. This database is a summary of various disciplines of brain understanding that can be interrelated with textural and graphic displays. The utility of graphics enhances the possible presentation of conceptual knowledge that has not before been possible. The three databases are not mutually exclusive but are represented as information transitions that extend from the laboratory to scientific exchange into the interpretive domain.

#### Criteria of System Components Selected

- 1.1.1 Computer - The computer selected was a Digital Equipment Corporation (DEC) MicroVAX II, configured as a Vaxstation II/GPX color graphics workstation. The MicroVAX has 11 Mbytes of core memory, 711 Mbytes of fast disk storage, 95 Mbytes of removable tape storage, 2 400 Kbyte floppy drives, an Ethernet controller for networking, and 24 serial lines. The GPX graphics workstation is a 8 bitplane, 1024 by 864 pixel format, subsystem with dedicated graphics controllers to relieve the CPU of intensive graphics computations.

- 1.1.2 Image Analysis System - Megavision's 1024XM system was selected for processing speed, programming flexibility, and large image format over other potential machines. In addition it was, at the inception of the contract, the only system that provided 1024 by 1024 pixel pipeline processing.
- 1.1.3 Operating System - UNIX was chosen as the operating system. UNIX, and its primary programming language C, best supported the image analysis subsystem and the GPX graphics workstation. The power and flexibility of the GPX workstation was tremendously increased with UNIX due to recent implementation of MIT's X-Windows software system.
- 1.1.4 Graphics - X-Windows, and its high level applications interface X-Tools, is as an increasingly universally accepted standard of graphics implemented on most computers. It provides for multitasking, multiuser, and multiwindowing graphics applications hitherto too demanding or complex.
- 1.1.5 Test, compare and select equipment for the methods of brain cell chemical contrast staining and its quantitative analysis.

Microscopes - The microscope that we are employing for computer-assisted quantitative microscopy is the Zeiss Axiophot, equipped with full fluorescence, bright field, phase, and Nomarski optics, and a highly advanced photographic system utilizing infinity optics. In addition, the Axiophot possesses a photographic system with two 35 mm ports and a video port simultaneously available to capture the image. The Axiophot also has a point light metering system which greatly improves the capacity for fluorescence photomicroscopy. The microscope was fitted with a Mertzhauser computer driven stage, capable of movements as fine as 1/10 of a micron, and with total travel of up to 10 cm (about 4 inches). In addition, Zeiss added a Z focus controller such that focusing could also be controlled by the computer. This is essential in the design of an automatic data acquisition microscope system. The stage arrived on 3/1/87 and controlling software for computerized mapping is under development.

#### System Description

We have combined the components listed above to create an integrated brain mapping system. The principal elements of the system are a host computer, image processor, cryomicrotomic workstation for RCI's, a macroscopic workstation for TSI's, and 3 higher resolution microscopic workstations with computer controlled stages also for TSI's. These are a large specimen TSI microscope, a fluorescence



microscope, and a conventional microscope. These components are integrated through customized user interfaces to expedite the flow of data through the image acquisition and image processing steps in order to provide digital graphic records of brain structures for quantitation. The maintenance or correction of image data to yield an accurate map of whole brain sections over a range of magnification is now fully achievable for rat and monkey brains, and partially achievable for human brains. As yet, however, incomplete components of the system hardware is constraining our capacity to obtain human brain data from all of the magnification levels. (See fig. 1.0-2).

1.2 Develop Software Routines for the computer-aided acquisition and analysis of tissue images [See Fig 1.0-3]

Software development occurred in a two-stage sequence:

I. First we had to develop a multi level UNIX-based image analysis software package which was able to address both low system levels, such that specific image processing and analysis steps could be rapidly achieved at the machine level. A higher system level was woven into the applications systems control in a way that there was no need to redesign the whole software program with each modification. Second, we needed to develop mathematical algorithms pertinent to the Brain Mapping Project. Once these principals were defined and the hardware options were understood, we developed application programs.

II. Next we had to refine the basic routing of procedures and then implement them into user interfaces to achieve the scientific goals of the project.

In Phase I, user interfaces were developed for image management and processing in the MegaVision and the Vax, as well as to control interactions between the two systems. This package is called UNIX Multiuser Image Processing Software, or uMIPS. uMIPS is a series of command programs that are designed to control the image processing environment, including any number of users or 1024XM systems, and the Mertzhauser stage on any number of microscopes. uMIPS was fully intended from the beginning to be implemented as an applications software, and has been now successfully implemented in 3 separate instances. The programs are UNIX-based, making them highly flexible and callable from nearly any level of the UNIX software hierarchy. The internal structuring of the programs was also such that embellishment would not involve rewriting major portions of the code.

Phase II included the development of the applications packages, SLICER, MAPPER, ABM EDITOR, COORDINATE RESTORER, AMENDER, MICROMAPPER and PRESENTER which are in part based on uMIPS [See Fig 1.0-4].

SLICER:

The sectioning and image acquisition software is called SLICE. This package provides an interface for users to easily employ UNIX control of the MegaVision system. These commands are specific to RCI acquisition. The program has been used successfully to obtain a series of block face images from the LKB microtome for a number of brains [See SOW 2.0]. Version I of SLICE was not highly sophisticated;

it did not incorporate much in the way of processing the images, but rather was a tool for the easy capturing of images. This was used to define the procedural steps and make first demonstrations of COMPUTER-AIDED ACQUISITION OF TISSUE IMAGES (SOW 1.2). It was also very important as a test for uMIPS, resulting in the satisfying conclusion that uMIPS is designed properly such that applications could be constructed easily and quickly for specific objectives.

SLICE has been consequently upgraded, and now includes methods for the automatic filenaming of images, automatic correction of non-uniform shading across the image field, and the automatic processing of the images through user-specified sequences [See Fig 1.0-5, 1.0-6].

#### MAPPER:

The analysis of blockface images (RCI's) and Tissue Section Images (TSI's) is achieved by a series of routines called MAPPER. The sequence represents a cascade of processes (figure 1.0-7,-8) that generate contours of image structural boundaries. The contours consist of coordinates that define a linked series of points, with vector dimensions defined by adjacent points. This series of points can be labeled to define a combined set of coordinates that make up the contours for the surface of each object in the section.

The sequence of operations seen in Figures 1.0-7,-8,-9 use Megavision routines to optimize the image and then define the edges. One of the first operations is to subtract the normal background eliminating the unevenness of the illumination inherent in the system. This process includes image normalization which remaps the density values from low contrast images so they cover the full 256 range of intensities. Once an image is normalized edge detection or thresholding routines are run. This threshold is mapped to a binary overlay channel for comparison of the threshold level to the image boundaries. An edge detector is used on this binary image to generate a set of pixels that define this boundary. These pixel lines are then thinned to a single pixel width. A line follower tracks the boundary from an arbitrarily defined point to extract a serial set of coordinates for pixels that comprise contour data.

The operator controls are shown in Figure 1.0-8 and consist of parameters settings, track ball control, image access control, and Megavision pixel processor operations. The detailed functions are listed in the Figure legend 1.0-8.

A number of approaches to the extraction of image boundaries have been attempted during the first year of the

project. These included a series of new algorithms called ROTATING BAR, HARALICK and CONTOUR FOLLOWER.

Detection of edges in the intensity picture is a primary task in our analysis step. A rough-cut (and fast) edge-detection and boundary generation can be done using the standard software provided by Megavision. This however suffers from accuracy and is not robust against factors such as non-uniform illumination across the image field, noisy observations etc. Our goal was to design an edge-detection and boundary extraction scheme which would work even under such adverse conditions, obtaining results consistent with the information in the image. The rotating bar and facet-model are edge detectors that use local intensity derivatives for finding edges, since an edge is nothing more than a local variation in pixel intensity. Both algorithms generate an "edgel" file that contains edge magnitude (strength) and edge direction for each pixel in the image. This information can then be used by an "intelligent" contour follower to produce a contour file in a vector format, where the contour lists are described in terms of the different points belonging to them.

The main element of the contour follower is the design of the successor function, which at any given point on the contour selects the function that is most likely to prolong the current contour. The choice is guided by the magnitude and direction associated with each edge pixel, and by such parameters as the look-ahead depth, the tolerance in direction, and the closest allowed distance to a neighbor contour. Both algorithms were implemented in C under the Microvax operating system. Work is currently under progress to design an efficient edge-detector (single pixel-wide edges) that will detect all the points on the boundary accurately and pass them to a 3-d graphics routine which generates the full model. We are investigating edge-detection algorithms sufficiently robust to detect edges at both high and low contrast.

#### MICROMAPPER:

We have created a micromapper-applications software package based on uMIPS called NEUROQUANT. This package was directed towards quantitative microneuroanatomic data acquisition and analysis. Using this interface, image frames captured from the microscope field were scanned by the computer to determine the location and relative amount of a specific chemical marker found at the cellular level. This was accomplished by measuring the transmitted optical index (optical density) of each cell and comparing this value between two populations of brains. This would have been a prohibitive task if done manually with a densitometer and film, due to user error and bias. NEUROQUANT functioned to process the images automatically, with only a small level of

interaction from the operator who instructed the computer as to which populations of cells were of interest. NEUROQUANT is presently being implemented with a more fully automatic method of localizing the cell bodies directly through a series of techniques based on intrinsic differences in texture and optical density among the various cell populations and on pattern recognition. NEUROQUANT will be used to obtain a database of cellular information and to create a library that can be displayed on other facets of the computer system, such as QUICKMAP (see below).

#### AMENDER:

The graphic contours extracted by digital means must occasionally be corrected due to blemishes in the preparation, or inadequacies of either the image or analysis. Correction operations must be interactively made but are greatly enhanced by the utility of the tools that provide for the operation. A number of Amending Programs have been developed to deal with different types of records. The first program appears in the Mapper Program where a facility is available to define and cut regions of the image from the binary thresholded selection. A second specialized editor is called ABM EDITOR. This program allows the operator to manipulate stored images and control editing operations (See Figure 1.0 9-10 for details).

A third editor, GPXedit was developed on the GPX terminal for recalling images and applying pixel manipulation of binary thresholds. Thus this program can interactively amend the results of edge detection. The display presents a scaled gray image of a whole section image, a partial display of the full scale gray image that is scrollable, and a partial display of the full scale binary image that is also scrollable.

Each of the scrollable images is divided into regions. A user clicks the mouse in a region and it is expanded into a bitmap editor. The mouse is then used to selectively erase or fill in pixels. The corrected image is then ready for contour following.

The fourth editor appears under the capabilities of Brainmate, where the image can be retrieved and modifications can be made to the contours that are interactively generated by the BRAINMATE INTERACTIVE MAPPER.

#### COORDINATE RESTORER:

This operator interface provides the tools to interactively restore the coordinates on the tissue section images to match those extracted from the RCI. (figure 1.0-11-12). The objectives are two-fold: 1) align sections in the same coordinate frame as the blockface records and 2) de-

distort the section image. The operation sequences that are effective in restoring the TSI to the RCI coordinates are currently performed interactively using tools operating directly in the Megavision environment. The functional sequence begins with the image intensity reversal so that the two images can be overlaid on the display and both can be viewed for interactive matching. The first operation is to translate a selected point on the TSI image to the same point represented by a defined structure of the RCI. The next step is to rotate the image so that structures fall along general XY coordinate axis. The image may still not match after this procedure because of distortions, if so, warping is required. (Note: at this time Megavision has not supplied the rotation procedure and thus rotations must be done interactively using a live-displayed image as an overlay to the "truth image" held in memory.) Warping is done interactively by operator location of match reference points. The translation, rotation and warping procedures may have to be done several times to achieve a best fit. Image processor controlled digital approaches to this operation are needed to achieve rapid execution of this procedure.

#### **PRESENTOR:**

This collection of programs includes various routines and packages which provide tools for plotting graphics of section atlases, displaying and plotting hardcopy images, image manipulation, wire frame display and hardcopy generation, solid image generation (CONTOURVIEW), as well as wireframing and rendering (An additional presentor, Movie.BYU, is to be implemented soon).

One of these interfaces, called QUICKMAP, utilizes the high speed graphics of the X Windows system. In general, QUICKMAP is used to recall standard wireframe models of brain atlases that have been stored into computer memory. Different aspects and magnifications of the brain may be presented on the screen. Naming handles called CAPITOLS have been coded into the model and represent a method to encode data onto a bounded location in various brain structures. The interface allows entry of SYMBOLIC or ICONIFIED data into these models and the association of the data with these CAPITOLS. Several forms of data may be entered. Examples are the cell types, the sizes, the numbers, the density of cellular information, actual images of representative tissue sections imported from the NEUROQUANT system, as well as textual types of information (e.g. comments or literature citations).

QUICKMAP is not an implementation of uMIPS, but rather another programmed level that is actually capable of calling uMIPS software. It is not designed to do image processing work, but can interface with such programs and is capable of

porting raw data to its symbolic representations.

#### Image Access:

We have created software that presents the scaled icons of images in a tabular format that is scrollable. This format graphically summarizes the contents of an image directory. These icons are then selected with the mouse to generate full displays, or to allow for further manipulation.

#### Image Display:

The GPX while running X Windows is a multiple windowing system. Unfortunately, the maximum screen display size is 1024 by 864, while our images are usually 1024 x 1024. We therefore have produced software for presenting images in smaller sized viewports, while providing the capability to scroll the image, resize the display window, or zoom selected regions.

#### Advanced Development:

Automated Brain Mapping (ABM) is an application designed for the capture (ABM SLICER), and after-capture processing (ABM EDITOR, ABM MAPPER), of the series of block face images needed to obtain the contour data. This interface is also based on uMIPS. Contour data consist of the actual lines that are defined by the neural structures contained within the images. The contour follower used in the ABM package is a rudimentary edge detector that we developed as a interim solution to getting edge information until ROTATING BAR can be perfected. ABM was applied to a series of rat block face images and processed for external surfaces and ventricular structures. The first version employed a non-adaptive thresholding technique and a series of cascading filters to isolate these contours. While not yet perfected, it demonstrates the ability to apply uMIPS to an application level, and the capacity of the computer system to prepare WHOLE RAT BRAIN ATLASES (Objective 2.1).

A method of organizing this large amount of data is needed at this stage. One of our software goals is the incorporation of a reasonable inference system for hierarchically and relationally describing and manipulating bodies of data. This software, for example, would be used for decorating brain atlas templates with neurosciences knowledge and making subsequent inferences. The current design is binary data modelling for defining system entities, attributes and relations.

Figure 1.0 - 1

## Overview

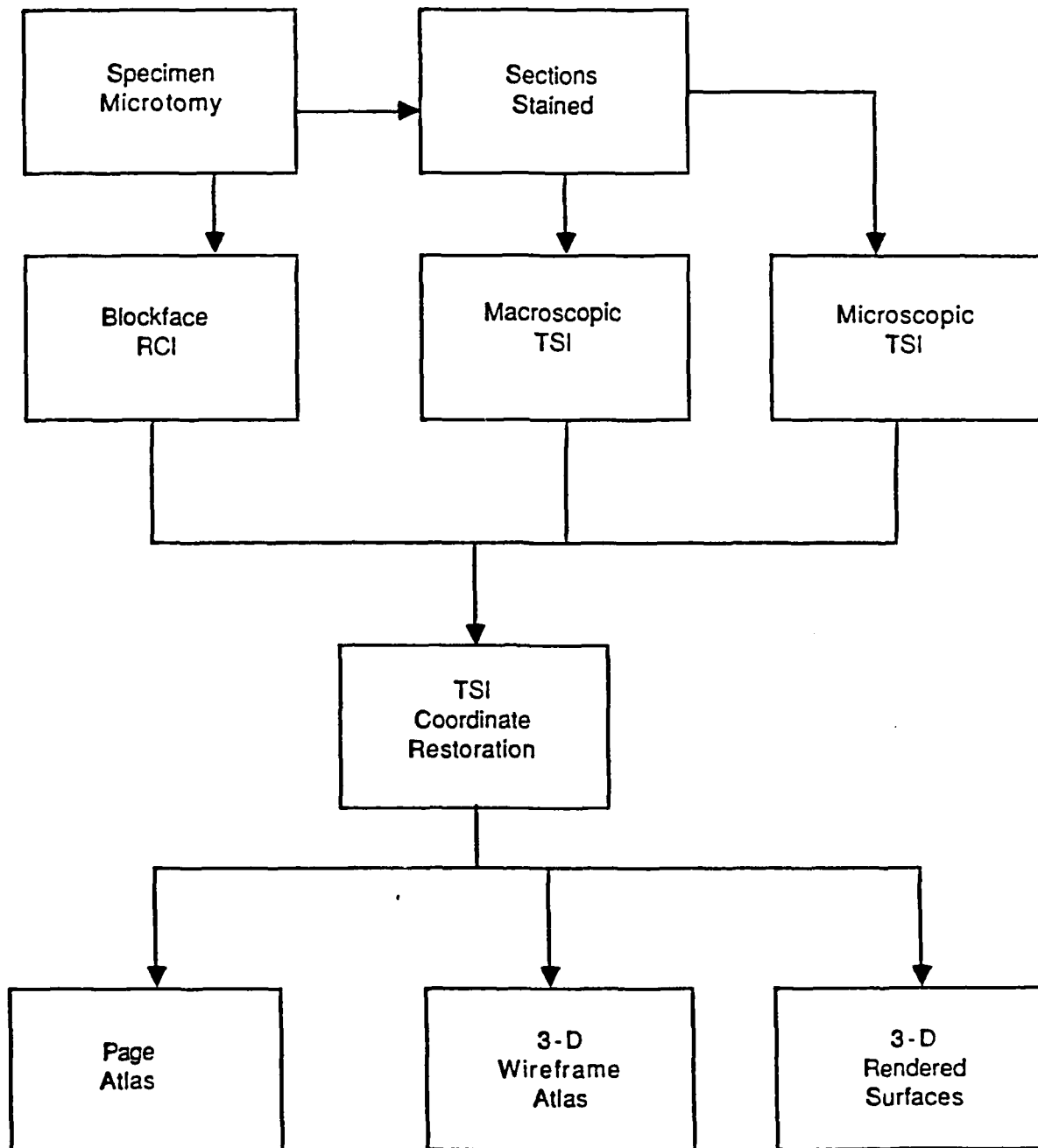




Figure 1.0 - 2

# Brain Mapping Hardware Configuration

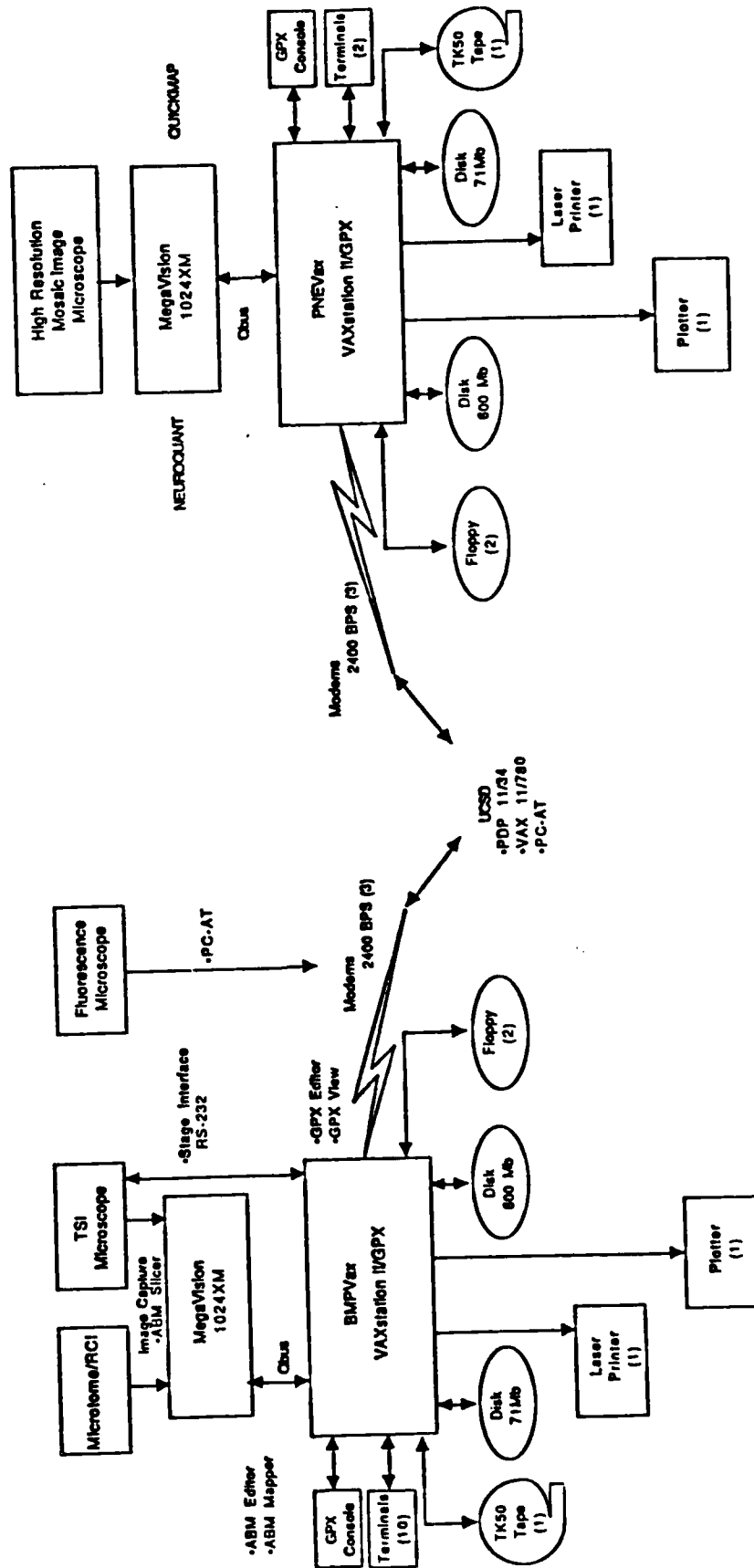


Figure 1.0 - 3

## Development of Brain Mapping Technology

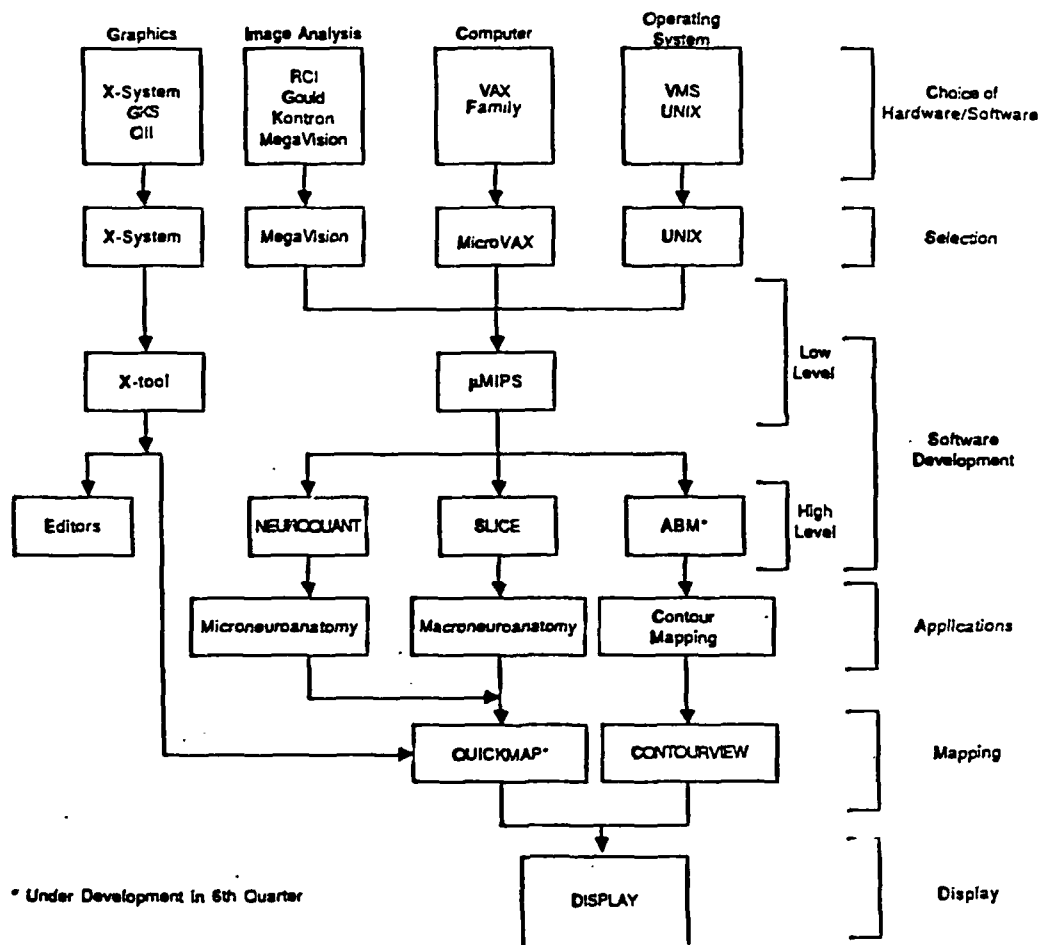
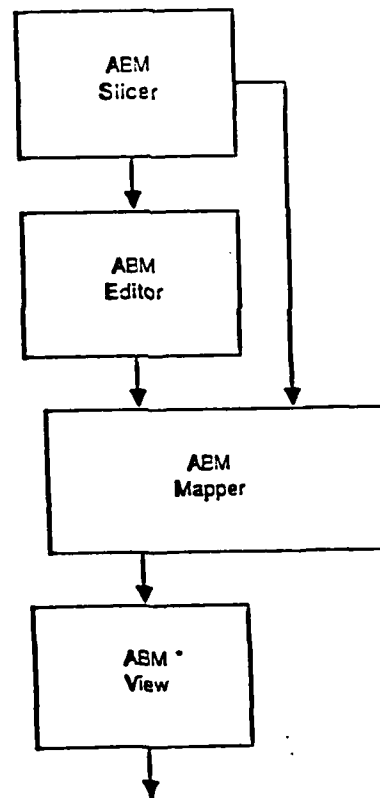


Figure 1.0 - 4

RCI



\*Under Development

Figure 1.0 - 5

## ABM Slicer

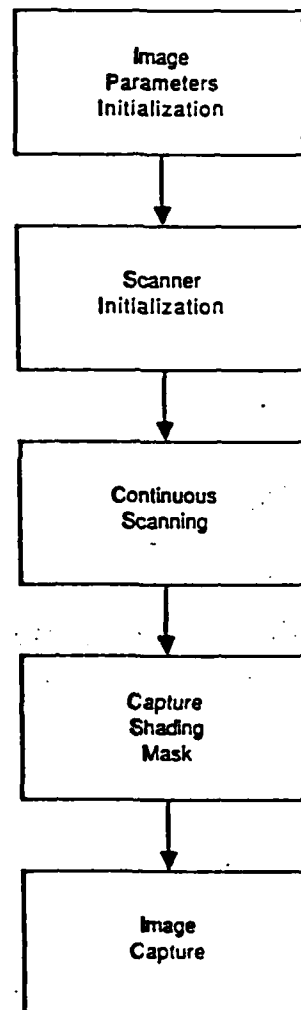


Figure 1.0 - 6

ABM SLICER V1.1  
(c) 1987 UCSD

- 0 - Exit slicer
- 1 - Initialize scanner
- 2 - Change/reset environment
- 3 - Capture shading mask
- 4 - Continuous scanning
- 5 - Capture image

Select ?

study test    dir /monkey/monkey2    images 18    time 12:00pm

The ABM Slicer program is designed for automatic acquisition of blockface images. In the first step, the camera is initialized by adjusting the analog circuits to provide optimum use of the analog-to-digital converter range. The working directory, root filename, current sectioning depth and increment may be changed with Option 2. Option 3 provides a continuous live image. A black surface image is captured for correcting non-uniform lighting using Option 4. In Option 5, images are then captured by accumulating and dividing to reduce noise. Automatic filenaming is used in storing the images.

Figure 1.0 - 7

## ABM Mapper

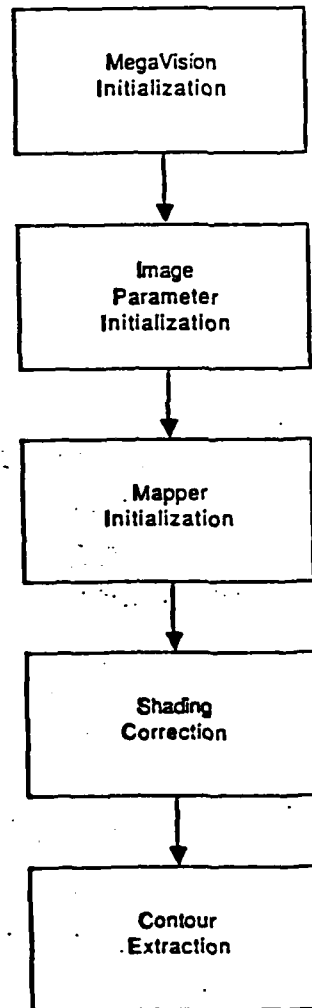


Figure 1.0 - 8

ABM MAPPER V1.1  
(c) 1987 UCSD

- 0 - Exit mapper
- 1 - Start/Stop megavision
- 2 - Re-initialize megavision
- 3 - Change/reset environment
- 4 - Initialize mapper
- 5 - Convert to gpx format
- 6 - Apply shading mask
- 7 - Produce contour

Select ?

```
srcdir /monkey/monkey3 (70) dstdir /monkey/monkey4 (3)
image hratl2400          megavision on             time 12:00pm
```

The ABM Mapper is designed to produce contours from the captured grey-level images. The MegaVision unit is turned off and on with Option 1. Option 2 resets the Megavision working environment to the original predetermined parameters. Option 3 allows the operator to change the working directory, root filename, depth of image in brain, sectioning increment and directory destination of the contoured image. Copies of the original grey-level images from one directory are placed into a temporary working directory in Option 4. Images in the working directory are converted to GPX format with Option 5. Option 6 corrects the images for non-uniform lighting using the pre-captured shading mask. In Option 7, contours are produced in vector format after user-interactive image editing and anatomical structure selection.

Figure 1.0 - 9

ABM  
Editor

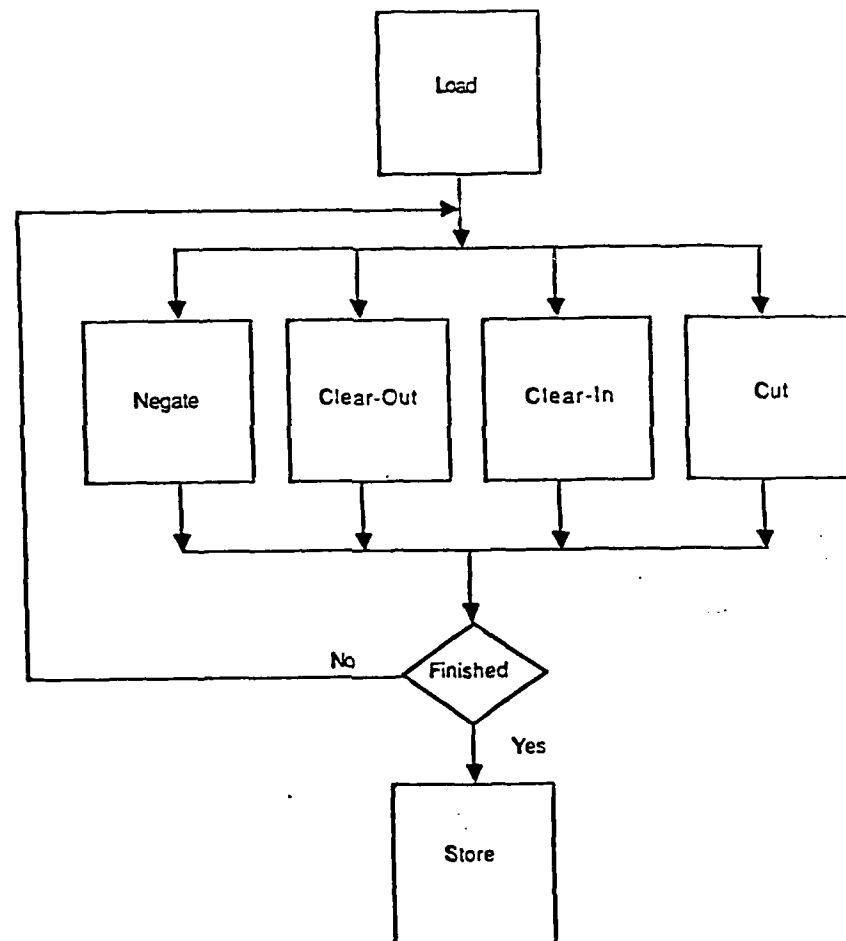




Figure 1.0 - 10

ABM EDITOR V1.1  
(c) 1987 UCSD

- 0 - Exit slicer
- 1 - Load image
- 2 - Save image
- 3 - Negate image
- 4 - Clear outside
- 5 - Clear inside
- 6 - Cut
- 7 - Erode image

Select ?

The ABM Editor is designed to allow operator-interactive editing of an image. The image is loaded from the Unix filesystem into a MegaVision memory using Option 1. At the conclusion of an editing session, the edited image is saved from the MegaVision memory into a file, using Option 2. Option 3 performs a logical negation of the image (pixelwise NOT operation). Option 4 erases everything outside the designated outline. Option 5 erases everything inside the designated outline. Option 6 allows the operator to separate anatomical objects from blockface artifacts. Black objects in the image are then smoothed using the erosion algorithm in Option 7.

Figure 1.0 ~ 11

### Tissue Section Image (TSI) Analysis

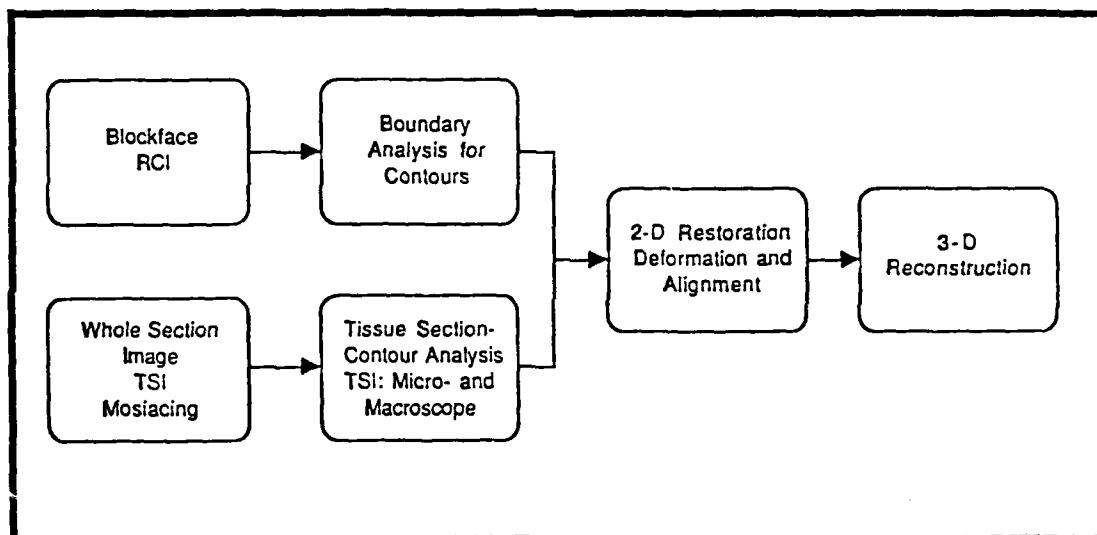


Figure 1.0 - 12

## MEGAVISION GEOMETRIC CORRECTION

1. Afmatch
  - a. exit
  - b. match control points
  - c. perform transformation
2. Transform
3. Tlist
4. Tstore
5. Trecall

The MegaVision geometric correction algorithm is used to align images so that one image may be sequentially registered with another. The first option, Afmatch, performs an affine transformation by matching control points in two images. One match of control points gives a translation. Two matches give a composite of rotation, uniform scaling, and translation. Three matches give a general affine transformation. The transformation parameters are then stored in a common data area so that they may later be used by other programs. Option 2, Transform, takes the transformation parameters stored in the common data area and applies them to an input image. The result is then placed in an output image. Option 3, Tlist, provides the operator with a list of the stored transformation parameters and any associated notes. Option 4, Tstore, takes the parameters determined by the affine transformation from the common data area and stores them in a user-named file with the extension, .PAR. Option 5, Trecall, takes the transformation parameters from a specified, stored file and loads them into the common data area for application to an image.

## 2.0 Acquire and Create (Morphometrically-Correct) Large Scale Brain Maps

### Description:

This section of the study is designed to develop and test the procedures of specimen preparation, image acquisition and image analysis through utilization of the block-face and tissue sections. The goal is to generate test brain atlases of three species by using the Tissue Section Image (TSI) and block-face images (Registration Control Images or RCIs) in combination to generate accurate spatial representations of the brain graphically. The software-hardware system design under Scope of Work 1.0 is being tested under this section and implemented for its utility at each of the three species. In the biological domain, a number of specific problems had to be addressed: 1) stabilization of brains in situ, 2) optimization and fixation of the coordinate plane for sectioning, 3) colorization for generating differentiation of internal structures, and 4) alignment of tissue from sectioning to achieve accurate three-dimensional atlases and reconstructions.

A number of factors had to be considered in order to achieve stabilization of the brain so that structures are maintained within 100-200  $\mu\text{m}$  of the in vivo condition. These included hardening types of fixatives, swelling and/or shrinkage that might result during preparation, brain sectioning in the skull or removal without distortion and a consistency of orientation between various brains during sectioning.

The production of symmetrical sections facilitates much of recording, aligning and analysis of images. A method of consistent orientation of the brain for sectioning was achieved by a brain holding device that secured the brain in relationship to an externally formed reference surface.

A third requirement was that at least the myelinated regions be separable from the grey matter by standard digital analysis hardware and software using videcon camera images. Digital frame methods for recording whole sections require sufficient density for object separation from the surrounding background and differentiation of internal structure. Staining methods were tested for generating specific densities that define the surface from the background and allow differentiation of myelin and cellular-neuropil zones.

The question of how to accurately align sections was solved by producing both block face images (RCI's) and stained sections by digital recording as single frames and as mosaics for higher resolution records. The block-face

image serves as the "truth image" for both aligning and warping the distorted section images. The rat in particular was and is being used as a test bed for development of this operation. Some delays in software by vendors, and therefore our inability to complete the procedures, has limited this operation.

Tests for various procedures were carried out in each category. These were done on the rat as much as possible in order to gain information for an array of approaches. The result has been that a definitive series of procedures has been brought together for addressing brain mapping using digital computer tools. These tools are now being used to generate brain "atlases" of generally agreed upon structures in three species for mapping the brain surface from sections.

# **I. Development of Protocols for Achieving Accurate Coordinates for 2-D and 3-D Brain Mapping**

## **A. Considerations and Procedures Attempted to Define Protocols**

Factors Involving Registration Control Imaging: Our own experimental results combined with a substantial amount of library searching has lead us to devise a method of preparation which we believe will overcome the many problems mentioned above. While no simple solutions were forthcoming, a powerful linkage of techniques was possible for preparing specimens for all three species in a way that yield a means to achieve digital analysis.

As mentioned under the description of the **Registration Control Image** (above), specimens must be prepared such that they are amenable to the collection of high-quality tissue sections. We have intensively investigated whole head cryomicrotomy as the initial approach to obtaining thin sections. What we have determined is that, while cryomicrotomy is excellent for the "cryoplaning" adequate for RCI's, it is insufficient for collecting preservable sections of 10 to 50  $\mu\text{m}$  in thickness that are needed for TSI micromapping.

The recommended method for collecting sections from large blockfaces of differentially resilient material is to affix a piece of self-adhesive plastic tape to the surface of the specimen and then to cut the section. This effectively yields a tissue slice that largely retains it's dimensional stability throughout the cutting procedure, and that can be subsequently transferred to a glass microscope slide. The problem with this approach is that cryosectioning induces a noticeable amount of chatter as the knife effectively "steps" it's way through the specimen. Since the surface of the block has been stablized by the tape, it

cannot follow the alternating compression-rarefactions experienced by the tissue lying on the plane of sectioning. In that frozen material is not particularly elastic, these differential stresses causes the section to crack. While this is not a significant problem for many histological investigations, it is unacceptable for TSI analysis. Clearly a way must be found to stabilize not only the surface of the specimen, but the subjacent tissue as well.

Collecting Sections for TSI's: The collection of sections has been delayed to ensure that the best possible RCI image can be produced. Collection of sections for TSI's is being tested on the rats. As mentioned, tissue sections are normally collected on tape and transferred to glass slides for histological staining. During this first year we have conducted experiments to elucidate the most effective method of obtaining non-morphometrically distorted thin sections for TSI analysis. To this end we have evaluated numerous forms of plastic tapes and adhesives. We currently favor 3M type 800 acetate-film tape, which is exceptionally dimensionally stable (15% stretch at the breaking point), moisture resistant, and possessing good adhesive qualities when applied to the frozen CMC blockface.

We have employed an  $\alpha$ -cyanoacrylate ester ("super-glue") to attach the tape-collected section to a glass slide, however, we are investigating the possibility of using standard histologically subbed slides instead. This will allow greater ease of positioning the specimen than can be obtained with commercially available glues. The recent development of albumin embedding has enabled easy section handling and now appears to be the method of choice.

Employment of Vital Dyes for Enhancement of the Blockface Preparation: In order to acquire image data in a digital form such that they can be manipulated by the computerized acquisition system, the primary sensor had to be a form of electro-optical transducer, such as a charge-coupled device or a vidicon tube, each of which has a unique spectral response curve. As can be seen in figure 2.0-1, such curves are highly non-linear. To further complicate matters the non-linearity of these transducers does not coincide with the spectral response of either photographic films or, more importantly, the human eye. Thus while the staining and histological preparative techniques have been created to provide maximal contrast when viewed by a human operator, they are not always optimal for machine imaging. This means that selecting stains to provide the white matter to gray matter contrast for the registration control images (RCI's) is a purely empirical process, and is not predictable from a study of the classical anatomical literature.

Thus, an important consideration was that in situ staining be done in a way to optimize imaging this

preparation. Furthermore, the stains used on the sections (TSI's) for defining structures at both the macroscopic and microscopic levels is important.

A major consideration in histological processing is that the procedure, whether vital dying, fixing, staining, or cryoprotecting must not only be accomplished in situ via vascular perfusion, but must induce a minimum of morphometric distortions, such as edema or shrinkage [SOW 1.3]. This immediately rules out many classic anatomical methods that depend on extensive stain incubation times, or require complex multi-step mordanting or defatting. Most of the analine dyes fall under this classification (Drury and Wallington, 1980). Finally, whatever anatomical methods are employed in situ for RCI data acquisition must not preclude the post-sectioning secondary processing necessary for tissue section image (TSI) analysis [SOW 1.4.2]. This secondary processing may include histochemical stains, immunolabeling, or preparation of small tissue blocks for electron microscopy.

A variety of compounds were tested for their ability to stain the gray vs. white matter in the brain well enough for video imaging. The class of compounds tested since it possessed sufficient permeability and could be simply applied to stain the entire brains in situ was the carmine-based dyes. Perhaps the main value of the carmine dyes is that they are ultimately simple to use, requiring no fixation, defatting, mordanting, etc. They also do not preclude subsequent fixation and counterstaining with other compounds. Lithium carmine (Orth's method, Gray, 1973) was used in the following example of a test for developing a suitable perfusion/staining protocol.

#### Procedure for Testing Carmine In Situ Staining using Rats:

Female (either 150-200 g Wistar or 200-250 g Sprague-Dawley) rats were anesthetised with Nembutol (50 mg/Kg) I.P. The thoracic cavity was opened by cutting the rib cage bilaterally approximately 2 cm from the mid-line. The diaphragm was spared as long as possible to maintain respiration. Following transection of the diaphragm, the rib cage was folded back rostrally, and the heart freed of connective tissue. The left ventricle was then cannulated with a dulled 27 gauge hypodermic needle connected via plastic tubing to a masterflex peristaltic pump set to deliver the perfusate. The right atrium was pierced with the tip of a number 11 scalpel blade and, simultaneously, the peristaltic pump started. Utilizing flow rates between 10 and 20 ml/min (depending on animal weight) the following solutions were introduced intracardially: 1. 25 ml of 0.15 M Potassium Phosphate ( $KPO_4$ ) buffer @ pH=7.2 (room temp)

2. 200 ml of Lithium carmine, made as follows: [Due to the uncertain composition of carmine, formulas are only

approximate, and must be adjusted for each batch of reagent.] The lithium carmine we employed was made as follows: 250ml H<sub>2</sub>O (distilled), 3.75 gm lithium carbonate, 8.0 gm carmine (Sigma). Mix together, boil for 10 min cool, filter, infuse at room temperature. (Note: Must be re-filtered prior to each use.)

3. A second 25 ml rinse with 0.15 M KPO<sub>4</sub> buffer (room temp)

4. 200 ml of fixative consisting of: 1% formaldehyde 2% glutaraldehyde in 0.15 M KPO<sub>4</sub> buffer (final pH=7.2) (4 °C)

5. 200 ml of KPO<sub>4</sub> buffered cryoprotectant [30% sucrose]

During the lithium carmine infusion the pumping rate was reduced to between 1 and 2 ml/min. This was to allow adequate time for the tissue to become evenly stained, while maintaining a sufficient flow rate to prevent vascular blockage from occurring.

Following cryoprotection, the head was skinned, the mandible exarticulated, and the upper dentation removed. The whole animal was covered with a damp towel, and placed on a bed of ice in a -20 °C freezer. This resulted in a very slow, even, freezing rate which prevented fracturing of the skull and attendant morphometric disruption.

Following freezing, the animal was decapitated with a saw and the head trimmed of extraneous muscle. The head was aligned relative to the microtome stage by hand, using stainless steel calibration tools to effect correct positioning. External alignment points were the auditory meatus and the inferior orbital rim. To orient for the taking of coronal sections, these four points were manipulated into a plane normal to the microtome stage and the head then frozen to the stage with carboxymethylcellulose (CMC). A mound of CMC was built up around the specimen to complete the embedding. In order to reduce the reflectance off the frozen CMC caused during RCI acquisition by the photoflood lamps, the CMC was dyed black with a water soluble ink. Finally the stage was aligned relative to the microtome knife-block using bubble-levels.

Results of Lithium Carmine Staining: Following the protocols above, we have found lithium carmine to moderately stain for differentiating neuropil from fiber tracts. We have also determined that more extended infusion times are required in order to uniformly distribute the dye. The partial diffusion of lithium carmine caused by inadequate incubation time results in a mottled appearance of the block-face, with the perivascular tissue more stained than that lying at greater distances. In addition, lithium carmine stains gray matter a purple-red, rather than a scarlet.



This color lies closer to the optimum spectral response of the MegaVision video camera and thus results in better contrast, and consequently better RCI's. Other carmines proved not only substantially more variable in their staining properties, but also yielded specimens with virtually no machine-readable contrast. As mentioned above, however, there are various spectral sensitivity drawbacks to the carmines.

Further Testing of Stains: To further enhance white and gray matter differentiation we explored other perfusion stains such as methylene blue, luxol fast blue, neutral red, and osmium tetroxide (See Fig. 2.0-2). In some of these cases we have also filled the vasculature with black, rather than red, latex for contrast enhancement. These substances were tested under a similar protocol as described for carmine with the exception of fixation prior to staining. Results showed staining with methylene blue (Ehrlich's method, Conn and Darrow, 1946) gave the most clear differentiation between gray and white matter in the brain as seen both with the human eye and with the MegaVision video camera. The standardized perfusion protocol developed is described in the following section.

#### B. Protocol of Choice for Preparation of Rat Brains

Standardized procedures arrived at to prepare the rat brain for sectioning as described below. The analysis of a number of solutions on various brains was described above has lead to the "preferred protocol" for the rat brain. The procedure found to be effective for fulfilling the criteria set forth above are as follows:

- 1). Anesthesia (Euresthesia type): 200mg/ KG intraperitoneally

- 2). Pre-perfusion Preparation: A perfusion board is used for maintaining a straight neural axis in the yaw, pitch and roll domains for the spinal cord medulla region. The heavily anesthetized rat is attached to a styrofoam sheet with 23 gauge hypodermic needles. Attention is paid to generating a slight stretching force between the incisor teeth and the tail. This is achieved by placing a short loop of suture over the dorsal incisors and over a needle that is lodged into the midline of the board. Force is then applied on the tail and it is pinned to the board. The sharp medulla-spinal cord neck flecture occurring in the supine position is reduced to a straight neural axis by placing a tube having 1.5 centimeter diameter at the nape of the neck so that it traverses the axis of the body. The forelimbs are then attached to the board in a spread-eagle manner by pinning each side with 1 1/2 inch long 23 ga needles.

3). Ventricular-Aortic Perfusion: The objective is to ensure a completely perfused brain that is undistorted by swelling or shrinkage. Three factors have been found to be important: a) cerebral vascular dilatation, b) a properly balanced wash and perfusion solution and c) controlled perfusion pressure. The first factor is overcome by a slight anoxia and critical timing of the onset of perfusion. The concentration of buffer components of the fixative and the amount of perfusion pressure are important considerations for maintaining the critical balance between swelling and shrinkage of the brain.

The thorax is opened by forming an abdominal-thoracic flap from the naval to the upper regions of the sternum. The rib cage is transected laterally on both sides. Care is taken so that the sternal and subclavian vessels are not encountered. The animal is observed for respiratory responses and the time is chosen to enter the aorta through the ventricle at the moment that the respiratory reflexes stop. A polyethylene tube that is larger than the aorta was thinned at the insertion end by pulling and the end was trimmed at 45 degrees. A permanent marker is used to color the tip of the canula and provide its direct visualization as it enters the aorta. Entry to the aorta is made by grasping the tip of the ventricles with a tissue forceps and the tip of the canula is directed through the left ventricular wall at the posterior side of the heart as it is lifted with the forceps. This direction in relationship to the body axis is in direct line with the aortic arch as it joins the left ventricle. A smooth insertion is followed by immediate application of pressure to the perfusion canula.

The critical points are that the pressure does not fall between the time of ventricular blood perfusion and the first of the fixation-sequence solutions and that the perfusion pressure is maintained in balance with the tonicity of the perfusion solutions. Thus, the vessels do not collapse or reconstrict because of a lack of pressure, while at the same time over pressure doesn't edematize the brain tissue and collapse the ventricles of the brain.

The right auricle is opened or the inferior vena cava is sectioned just as the perfusion pressure builds. Twenty to thirty cc's of a wash solution is applied through a 60 cc plastic syringe under controlled pressure. This is immediately followed with the fixative by a valve directing the fluid from a reservoir of fixative. This entire procedure is done at 120 mm Hg. The canula is tied in the aorta by a loop of 00 suture and the perfusion then continues undisturbed for 20 minutes. This administration is followed immediately by a solution of methylene blue for staining the gray matter. A second valve in series on the perfusion line is attached to a reservoir of this dye and the valves are

simultaneously switched from fix to the dye without pressure changes.

Perfusion Solutions: Three solutions are applied at the time of perfusion (all solutions are maintained at 40-45° C):

Wash Solution

Fixation Solution

1% paraformaldehyde 1% Glutaraldehyde

Methylene Blue

0.025% methylene blue 0.25 M Phosphate Buffer 0.03% Sodium Chloride

5). Cryoprotection for Whole Brain Embedding: A solution of 17.5% sucrose is used to immerse the brain for 24 - 48 hours. This sucrose concentration is carried into the embedding medium to prevent ice crystals in the embedding medium surrounding the brain.

6). Protocol for orientation of the brain to the microtome, stabilization of sections for collection and enhancement of contrast of the brain surface in relationship to a uniform light absorbing background.

A number of problems of sectioning were solved by development of a method for orientation of the brain through an embedding procedure. The main objective was to provide a consistent plane of section for each species of brain that was being sectioned ex situ. The adaptation of the procedure for background coloration allowed a significant increase in contrast between the brain surface and the surrounding regions. The same material stabilized the sections so that all of the island fragments remained in their proper relationship to the major brain mass and are not lost in transfer of the sections. This procedure also provided a means to more readily handle the large number of sections involved in the TSI operations.

The procedure for embedding the brain was a modification of the Albumin/Gelatin method that was needed to solve problems associated with sectioning whole frozen brains. The brain was soaked in 17.5% sucrose for 24-48 hrs and then placed in the egg albumin/gelatin mixture overnight. This solution was modified in two ways. First, india ink was added to the mixture to generate a high contrast medium. The large size of these particles do not allow penetration into the brain tissue. The second change was that the albumin/gelatin was prepared to contain 15% sucrose. The purpose of this was that ice crystals even though small generate a reflective mirror effect of the light sources that are projected on to the image. The very small ice crystals

that are able to develop in the brain and in the embedding block material are reduced to sizes that are not more than a pixel in size.

A specialized "brainholder" was used to mold the albumin/gelatin block with surfaces that were true to the orientation of the brain. This allowed the brain to be held in a consistent coordinate domain while the albumin/gelatin mixture was hardened around the brain. The block surfaces provide both a contact with the microtome cutting platform and a reference for brain rotation alignment to the knife. These serve to center the brain in the camera frame and optimally utilize the frame digitization area.

A metal device was fabricated for supporting the brain on three small contact points while the embedding medium is hardened. This maintains the brain orientation at the center of the rectangular-box mold and provides a reference for neural axis roll. This metal jig provides three reference surfaces in relation to the three points of contact with the brain. The metal forms the two ends of the box and one side, and is fabricated as a U-shape of polished aluminum. Three conical posts are attached to the side component so that one contacts the pons and is placed along the midline between the two end plates of the device and the other two are placed laterally for contacting the temporal lobes. The placement of the posts in the form of a triangle was made so that the tips of the olfactory bulbs and the spinal-cord medulla are equidistant from the ends of the box. These posts define roll and pitch of the brain in the rostral-caudal neuroaxis. The yaw is restricted by two 30 gauge needles that enter ports formed of tubes that are at the midline of the two endplates and are directly over the olfactory bulbs and at the central canal of the cord-medulla junction. The needle at the cord slightly penetrates the cord while the needle is only placed between the olfactory bulbs. Once the embedding medium is hard, sides fashioned of tape are removed along with the needles. The brain pops free of the mold as it is highly polished and waxed to provide easy release. The holes left by the posts are filled with the embedding medium.

Embedding medium: This solution consists of 28% egg albumin, .5% gelatin, 15% sucrose, and 1% india ink. At the time of embedding, nanograms of glutaraldehyde are added per cc of solution to denature the proteins and thereby stabilize the medium as a rigid gel. A diagram of the current standard specimen preparation from perfusion to alignment in the microtome is shown in figure 2.0.

7). Physical Sectioning: Video Imaging of the Block-face. CMC embedded rat brains destined for RCI only were sectioned in the LKB cryomicrotome at  $-30^{\circ}\text{C}$  with a  $35^{\circ}$  angle knife. The section thickness was  $5\mu\text{m}$ , with RCI's acquired

at 40 $\mu$ m intervals. Later, albumin embedded rat brains destined for RCI, as well as TSI analysis were sectioned at -10°C with a 20° angle knife. The section thickness was 50 $\mu$ m, with RCIs acquired at 200 $\mu$ m intervals. Tissue collection was found to be effective using the second set of sectioning parameters.

The video camera used to obtain the RCI's was a 1024X1024 pixel MegaVision videcon-type camera conjoined with a Tamron 70-210 mm macro-zoom lens. This system was mounted on a custom built gantry, designed to allow sufficient flexibility in camera positioning to accommodate heads from all three species (fig. 2.0-4). This arrangement also allowed us to adjust the focal length such that the desired region of the blockface (the CNS) filled the entire field. This provided a theoretical maximum point-to-point resolution of approximately 25 $\mu$ m on a coronal section, assuming a 25 mm cross section.

The blockface was illuminated with two 75 watt color-corrected tungsten-halogen lamps (Sunbeam Examatrons) producing 2500 footcandles, measured at the blockface (a distance of approximately 15 cm). The light was passed through diffusion filters to provide even illumination, and through parallel-polarized filters to reduce glare. The lamps were mounted directly to the camera gantry, which was in turn attached to the knife pillars, this ensured that the angle of illumination (empirically determined as that angle causing the least reflection--generally between 30 and 45 degrees from parallel to the blockface) would remain constant throughout the sectioning process.

To preclude spurious internal reflections from degrading the image, a custom photographic shroud made of black cloth was constructed. This unit is essentially conical surrounding the specimen stage and tapering upward to a small aperture, through which the lens is inserted. Additional ports on the sides admit the face-plates of the photoflood lamps.

The actual acquisition of each RCI frame was accomplished by electronically averaging 128 or 256 accumulated blockface images (using a MegaVision acquisition algorithm). This enhanced the raw image by averaging out any asynchronous noise. Following acquisition, each image was further enhanced using a MegaVision processing tool ("Sharpen", which degrades the gray scale thereby making the edges more distinct), and stored as a 1 megabyte disk file on the MicroVax.

8). Video Registration Control      Imaging: [SOW  
1.1, 1.2 and 1.3] We have obtained a full series of  
sagittal rat RCI's (345 frames at 40  $\mu$ m intervals) [SOW  
2.1 and 2.1.4] utilizing the Circon camera and

fluorescent lighting. These results enabled us to design the current video RCI system, which incorporates the MegaVision camera and tungsten-halogen lamps. Utilizing this system we have sectioned three rats (two sagittal, one coronal) and are now able to obtain sequential series RCI's with better than 30  $\mu$ m resolution.

Work with the MegaVision image processing algorithms has resulted in our now being able to produce stable high contrast images that are further processed by our own (ABM) edge detection and contour following programs. The results of these computer operations are the brain structure outlines that form the heart of the wire frame atlases.

## II. Whole Monkey Brain RCI-TSI Recording

### Method of brain preparation

Cynomolgus monkeys were deeply anesthetized with ketamine hydrochloride (25mg/kg, i.m.) and pentobarbital sodium (10 mg/kg i.p.). Each monkey was intubated with a 3.0 or 3.5 pediatric size endotracheal tube and mechanically ventilated with 100% oxygen. After the chest was opened and the heart exposed, 1.0-2.0 ml of 1% aqueous sodium nitrite was injected into the left ventricle. The descending aorta was clamped and the animal perfused transcatheterially with cold 1% paraformaldehyde in PBS for 30-60 seconds. Picric acid was introduced to the vasculature and allowed to flow for 30 minutes. Carmine staining was followed by perfusion with 4% paraformaldehyde in PBS for 8-10 minutes. Cryoprotectant consisting of 30% sucrose followed fixation. The lower jaw and upper dentation were removed and the remaining head was embedded in CMC. [Future monkey material will be stained with methylene blue following fixation. It will then be embedded in albumin/gelatin. See Fig 2.0-5].

## III. Whole Human Brain Preparation

The goal was to generate adequate human brain preparations that could be used in the giant cryomicrotome for producing RCI images. All specimens were obtained from the University of California, San Diego, Willard Body Program. Heads were selected based on several criteria, including 1. No known neurological or psychiatric disorders, 2. No metastatic carcinomata of types likely to invade the CNS, 3. No known vascular damage, 4. Specimens with intact eyes were preferred. Of the potential specimens from this pool we selected those heads that had a horizontal width in the coronal plane of 140 mm or less. This being the maximal size that can be accommodated by the cryomicrotome.

The head was shaved, washed and placed on a head-holder. The thoracic cavity was opened and the lungs removed. The sub clavian arteries were transected and the descending vessels (supplying the arms) ligated. The sub clavian arteries were then catheterized with 3/8 inch O.D. tygon tubing connected through a dual-head Harvard peristaltic pump to the perfusion solutions. The same sequence of solutions was employed with the human as with the rat (above). The differences were that the flow rate was 50 ml/min during all phases of the perfusion and that the flow rate was reduced to 0 during the carmine incubation. One final difference was that picrocarmine (Gray, 1973) was used in place of lithium carmine. [The reason for this was that we had not yet determined the substantive advantages of lithium carmine at the time of the human preparations. Future human material will be prepared utilizing either lithium carmine or Methylene Blue. See Fig 2.0-5.] Finally the vasculature was filled with red latex (Wards Biological Supply) to which a small quantity of Barium Sulfate (Esophotrast) had been added. This allowed the pre-capillary vasculature to be imaged by computerized tomography.

Following latex infusion the neck was opened in such a fashion to create internal pouches which were extended completely around the vertebral column. These pouches were packed with powdered dry-ice which was further cooled with liquid nitrogen. Care was taken to ensure that the base of the skull did not come in contact with the coolant, this in order to prevent fracturing the skull because of too-rapid freezing.

During the 2 hours required to fully freeze the neck and spinal cord, the head was further prepared for microtomy. This included exarticulation of the jaw and removal of the upper dentation. This latter was accomplished by hand sawing along a plane parallel to the base of the brain, essentially following the roof of the mouth. When spinal freezing was complete, the head was decapitated at the level of C<sub>1</sub>. The head, fixed but not yet frozen, can be examined by MRI or CT [See Fig 2.0-3]. Following these virtual sectioning examinations, the head is covered with a damp (not soaking) towel, and positioned in an orthogonal styrofoam box with the inferior orbital-auditory meatus plane set perpendicular to the base of the box. The head is glued in position with 2-3 cm of CMC, and placed in a -20 °C freezer for 24-48 hours. During this time additional CMC is added to the box in increments such that at the end of the freezing period the head is fully embedded in the sectioning medium. The box is removed and the block trimmed for final sectioning with a general-purpose industrial band saw.

We have now obtained a "library" of Human heads which have been perfused and frozen according to the protocol listed in Methods. In all, eight heads have been prepared

in this manner and are awaiting sectioning. These specimens will be sectioned by cryoplaning to generate the preliminary RCI human atlas.

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FIGURE 2.0 - 1

TYPICAL SPECTRAL SENSITIVITY CHARACTERISTIC

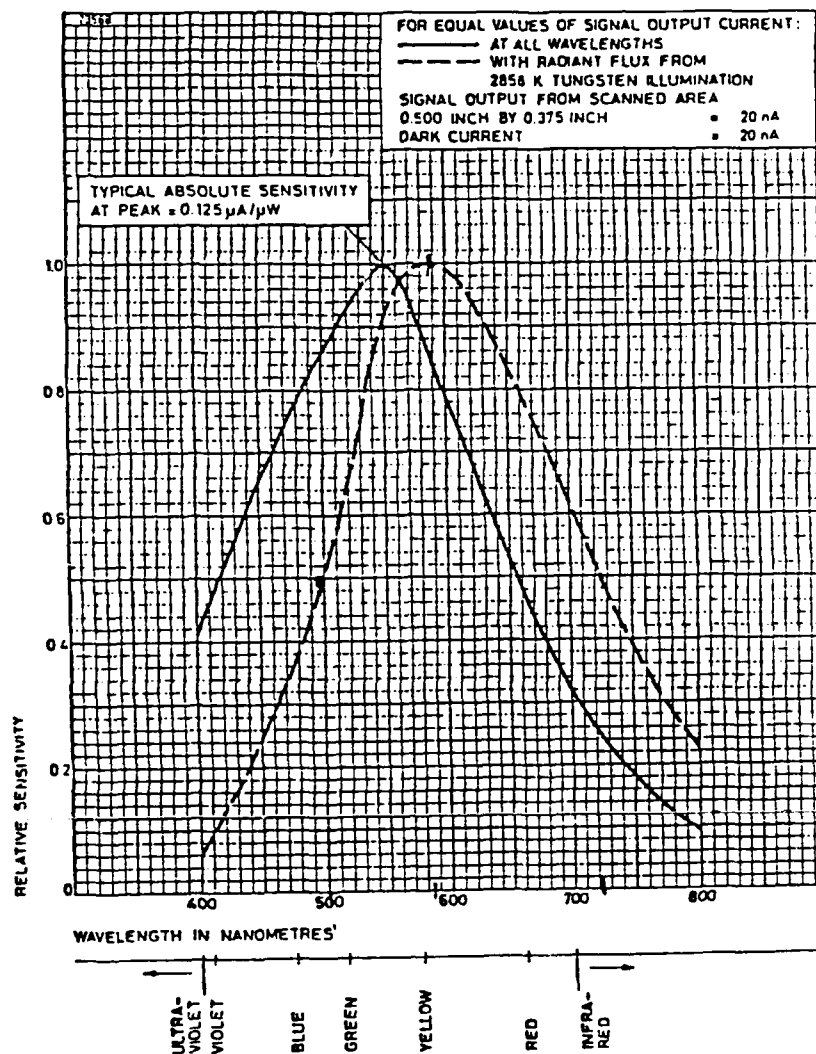


FIGURE 2.0 - 1

(Top) Spectral response curve of the Circon CCD camera. Note the substantial fall-off of sensitivity in those wavelengths associated with picrorcarmine dyes (680nm). (Bottom) Characteristic spectral response of MegaVision camera. Note that with tungsten illumination (dashed line) the red component of the carmine stain (the gray matter) generates nearly 80% max output, while the yellow component (white matter) generates almost exactly 100%. This is a much greater degree of differentiation than available from the Circon CCD camera, and partly explains the improved contrast we see with the MegaVision-lithiumcarmine combination.

FIGURE 2.0 - 2

# Development of Perfusion Protocols

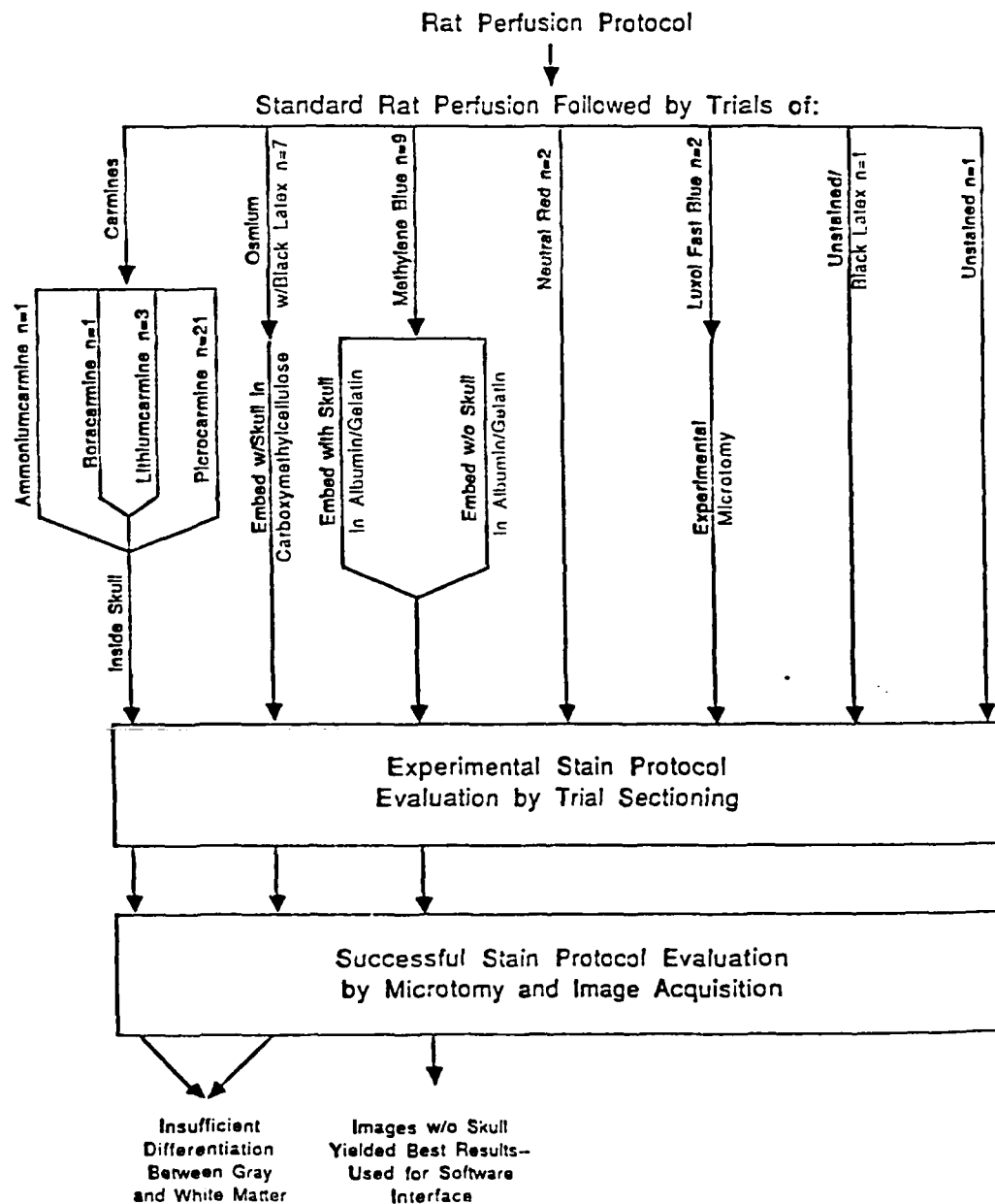


FIGURE 2.0 - 3

## Specimen Preparation

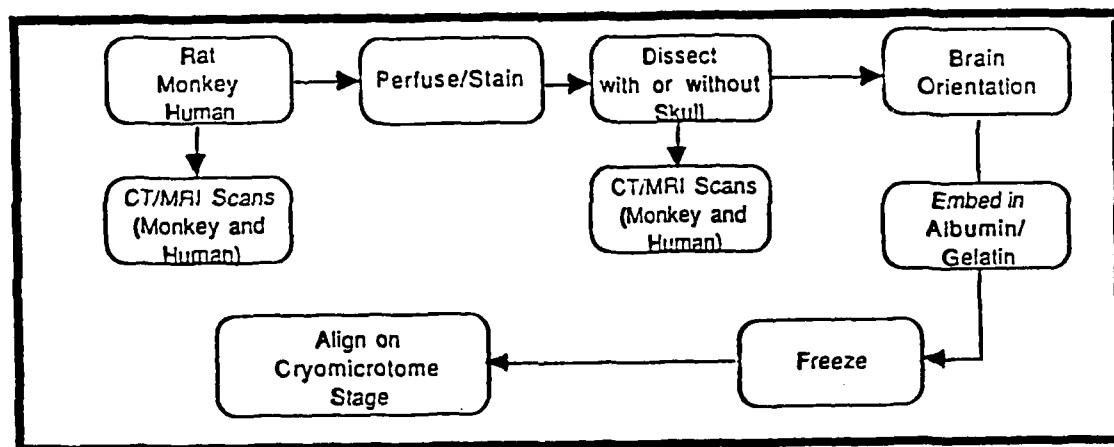


FIGURE 2.0 - 4

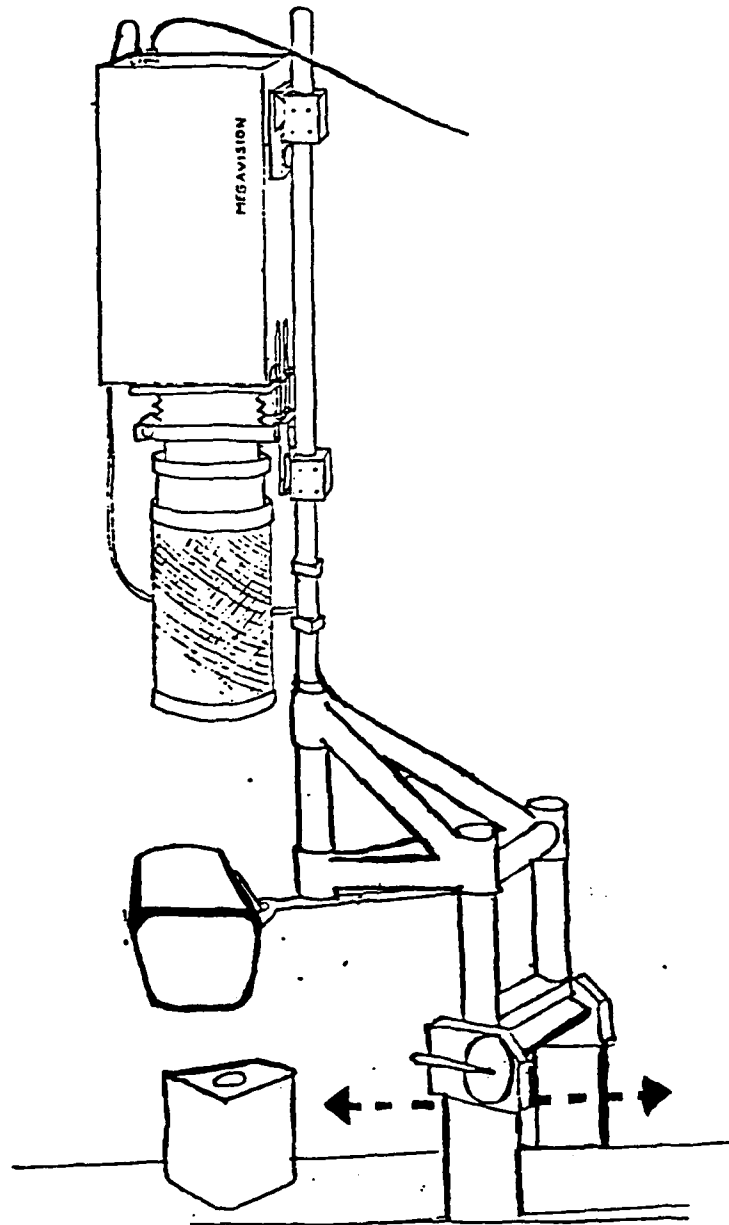
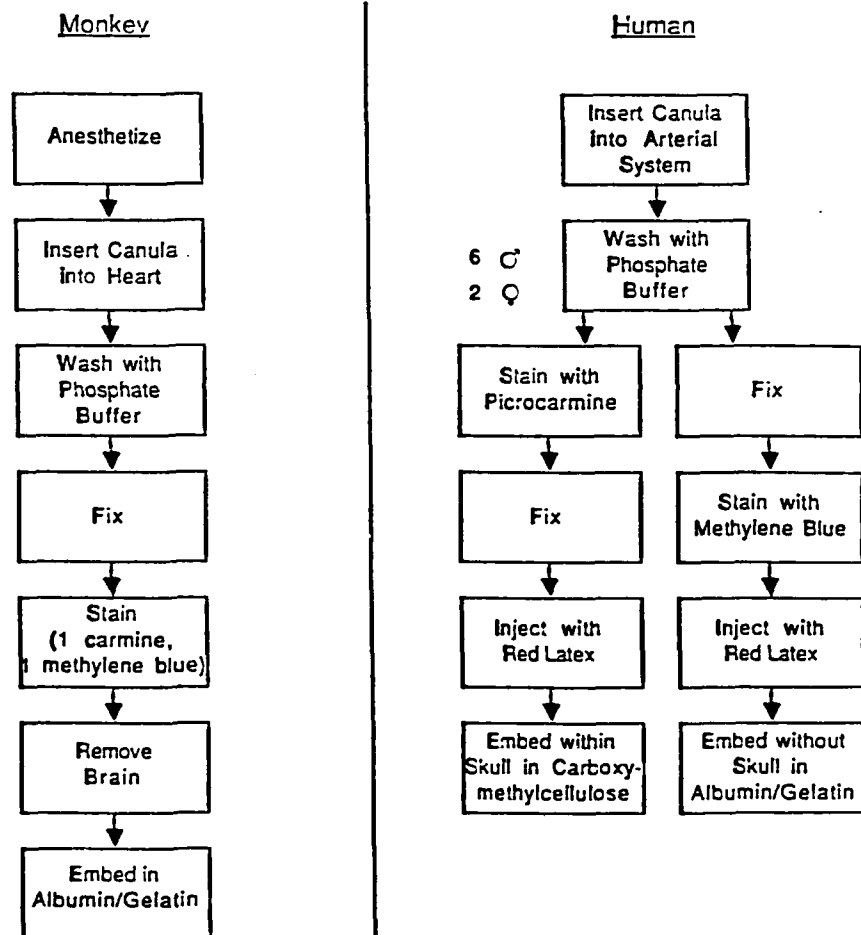


FIGURE 2.0 - 5

## Primate Perfusion Protocol



### 3.0 Acquire and Create Selected Sub-structure Brain Maps

#### Description:

The objective of this section was to develop a means to carry out subregional analysis for information from stains and reactions that localize specific cell types and axon projections. The approach was to define localization as contours and reference points mapped to specific regions of the brain so that data could be readily placed in the context of the RCI-TSI Maps generating accurately defined coordinates within the surrounding structures. Three species are represented for this testing analysis on the brain: rat, monkey and human, while the pigeon was the ideal choice for the detail of the nerve net retina.

In the rat, labeling of cell somata or axon populations included acetylcholine, catecholamine (tyrosine hydroxylase), and Substance P. For the monkey, the neocortex was selected to attempt a first run mapping of identified regions for specifically identified neurons. Two immunologically-identified cell types were chosen for regional mapping of the cortex in relation to cortical regions readily identifiable in Nissl preparations. The immuno-staining for /fBsomatostatin/fR has been done for a large number of areas of the cortex. As the RCI-TSI page-map of monkey data is produced, the labeled data can be registered to the page maps and 3D reconstructions.

A third area of study was the mapping of immuno-labeled types of amacrine and ganglion cells in the retina. Here fluorescence labels of different subtypes of cells were defined and placed in maps. A fourth regional mapping was done on the human thalamus for cytological definition of the nuclei. These were contoured from cellular stained sections in the Yakovlev Collection.

#### 3.1 Rat Immunological Labels for Neurotransmitters.

**Immunocytochemistry:** The goal is to map the locations of all the brain ACH neurons, projection site of Substance P on the brain stem and the localization of throsine hydroxylase (TH) cells throughout the brain.

The immunocytochemical methods used to map the ChAT-containing cell bodies are modifications of the techniques that were acquired by the investigator over a number of years of research. These methods are essentially the same for all three substances related to these transmitters. The following section describes the ChAT method as it applies to all these neurotransmitter systems.

**Fixation:** Male Sprague-Dawley rats were pretreated with heparin sodium (10 mg/kg) 15 minutes prior to sacrifice.

Subsequently, the rats were anesthetized with pentobarbital (60 mg/kg) and then perfused through the heart with 4% paraformaldehyde in 0.1M phosphate buffer at pH 7.4 (100 cc). This was followed by a mixture of 4% paraformaldehyde and 0.15% glutaraldehyde (400 cc). Following a six minute postfix in situ, the brains were removed and coronal slices 4-7  $\mu$ m thick were placed in paraformaldehyde overnight. In addition, we have been investigating the use of high glutaraldehyde mixtures as a primary fixative. To date we have been able also to obtain excellent ChAT and TH immunolabeling with glutaraldehyde concentrations as high as 2.5% (paraformaldehyde 2 - 4%). At these higher concentrations, however, the tissue needs to be pretreated first with 1% sodium borohydride (diluted in 0.1M phosphate buffer) for 30 minutes.

Sectioning: Immediately following the postfixation period, the tissue was sectioned on a vibrating microtome (Vibratome) at 40  $\mu$ m. The entire rostrocaudal extent of rat brain was sectioned. Individual tissue sections were collected in order and stored at -20°C in 24 well sterile tissue culture plates (Falcon) containing cryoprotectant solution (glycerol/ethylene glycol/phosphate buffer). It has been our experience that tissue sections can be stored for months in this solution without any effect on the quality of the immunolabeling. A freezer in the laboratory of the P.I. is dedicated to the storage of tissue sections.

Alternatively, for tissue sections processed solely for light microscopy, we find that sectioning on a sliding microtome is far more convenient than Vibratome sectioning and produces the same results. This approach is applicable to the brain mapping procedure where the blockface is recorded. Unfortunately, the brain mapping system was not yet operational during the time that the immunological material was prepared. When this latter method of sectioning is used, the tissue blocks are pretreated in a 17.5% sucrose solution for 2 days. These brains are then embedded in albumin/gelatin by the method described above for S.O.W. 2.0.

Immunolabeling Procedure: Our immunolabeling procedure was adapted from the method of Hsu et al., (1981). First, our tissue sections are removed from the cryoprotectant and rinsed five times for 10 minutes each in 0.1 M phosphate buffer. Subsequently, the tissue sections undergo the following series of steps:

- (a) Three rinses 10 min each with 0.1M Tris-saline containing 0.25% Triton X-100.
- (b) Pretreatment of tissue sections for 30 min with 3% goat serum in 0.1M Tris-saline containing 0.25% Triton X-

100.

- (c) Two 10 min rinses with Tris-saline containing 0.25% Triton X-100.
- (d) 24 hour incubation (room temperature) with antiserum to ChAT or control serum. The ChAT antibody is diluted 1:1500 with 0.1M Tris-saline containing 1% goat serum and 0.25% Triton X-100.
- (e) Two rinses with Tris-saline containing 1% goat serum for 10 min each.
- (f) Incubation for 1 hour with biotinylated goat anti-rabbit (Vector Laboratories) diluted according to their directions (i.e. 1 drop per 10ml of Tris-saline containing 1% goat serum).
- (g) Rinse as in (e).
- (h) Incubation for 1 hour with ABC complex (Vector Laboratories) diluted according to their protocol (i.e. 2 drops of A and 2 drops of B in 10ml of Tris-saline containing 1% goat serum). Technical note: The 1% goat serum in this final incubation is stated by Vector to dramatically reduce the intensity of immunolabeling. After repeated trials we have found this not to be the case, but rather that it improves the quality of our immunolabeled profiles.
- (i) Rinse as in (e).
- (j) Treatment for 15 min with 0.05% solution of 3-3' diaminobenzidine and 0.01% hydrogen peroxide plus 75  $\mu$ l of 8% nickel chloride/150 ml of solution.

Subsequently, the sections are rinsed in phosphate buffer, mounted onto glass slides, dehydrated in alcohol, coverslipped with Permount and examined with a Zeiss photomicroscope.

Control sections: Control serum consisted of either preimmune serum or preabsorbed antisera (5.5  $\mu$ g of ChAT/50  $\mu$ l if 1:200 diluted anti-ChAT). Specificity was judged by the absence of immunolabeling when either of these two control sera was substituted for the primary antiserum.

#### Preparation of Antiserum:

At present we have four different antibodies against choline acetyltransferase (ChAT). Two of these are monoclonal antibodies derived from rat and two are polyclonal sera raised in rabbits. Each have been aliquoted into small fractions and are frozen at 70°C. Although all antisera



provide excellent results, we for the most part use the antiserum generated by Drs. Hersh and Bruce (University of Texas Health Science Center, Dallas, Texas. This antiserum has been in use in our laboratory for over two years and provides very reliable and consistent results.

Choline Acetyltransferase (polyclonal): Polyclonal antiserum against ChAT was obtained from Drs. Gordon Bruce and Louis B. Hersh (Dept. of Biochemistry, University of Texas Health Center, Dallas, Texas). Details of the production and characterization of this antiserum have been recently described as follows [Bruce et al., 1985]. In brief, 150  $\mu$ g of purified human placental ChAT was emulsified in complete Freund's adjuvant and injected directly into the popliteal lymph nodes of New Zealand white rabbits. After 4 weeks a booster injection of 150  $\mu$ g of protein emulsified in complete adjuvant was given in the thick part of the skin above the scapula and neck region, followed by a second boost after an additional 21 days. Seven days after the last injection the rabbit was bled and the immunoglobulin fraction was purified on DEAE Cellulose. The polyclonal anti-human placental ChAT antibody was shown to react monospecifically with ChAT. In addition, our recent immunohistochemical studies demonstrated that the distribution and general cytological features of cholinergic neurons immunolabeled with two different monoclonal antibodies are similar.

Substance P (SP): The anti-substance P antisera was obtained from Accurate Chemicals. The production and characterization of this monoclonal antibody, which is the product of fusion of mouse myeloma cells with hyperimmune rat spleen cells, has been reported. The procedure we used is the same as for ChAT with substitution of this antibody [Cuello et al., 1979].

Tyrosine Hydroxylase (TH): Details of the methods of purification for tyrosine hydroxylase as well as production of specific antiserum and criteria of specificity have been described in detail by JOH et. al., 1973. In brief, the enzyme was partially purified from bovine adrenal medulla by a combination of precipitation with ammonium sulfate and column chromatography. The partially purified enzyme was isolated by disc-gel electrophoresis and injected with Freund's complete adjuvant into rabbits for the production of specific antiserum. Specificity of the antiserum was determined by selective immunoprecipitation and inhibition of enzyme activity by tyrosine hydroxylase. The method for reacting this antibody is the same as for ChAT.

#### Mapping Results:

To date, preparations have been generated for all three substances as indicated by direct or indirect immunoreactants using sections through the entire rat brain. For the

acetylcholine, the ChAT enzyme immunoreaction is complete for the entire brain. Sections were recorded as point loci formats for each cell at 600 $\mu$  separations. These are ready to be placed on a rat wire frame atlas.

Substance P mapping of 200 $\mu$  separated brain stem sections (where it is found) has been recorded for a single rat brain. These maps are of terminals and fibers that form a plexus over various regions of the medulla. The recording of these data are being contoured for areas that define zones. The contours will be assembled in the rat page atlas and wire frame representations for comparative display of structures with labels.

The TH labeling is completed for sections and is waiting to be recorded.

3.2 Neurochemical cell maps of primate cortex. Using the rapid, precise and quantitative microscope analysis subroutines EMMA, developed at SCRF under this contract, two well established chemical markers were analyzed quantitatively at the cellular level and compared across a wide selection of cytoarchitectonically identified neocortical regions.

The two chemical markers selected were: 1) the neuropeptide somatostatin, a putative neurotransmitter chemical employed by a large proportion of the non-pyramidal, intrinsic neocortical neurons; 2) tyrosine hydroxylase, an enzyme found exclusively within the nerve fibers of the two principle catecholamine transmitter systems in the forebrain and are shown experimentally by our data in old world monkeys to represent largely the dopamine system, a fiber system considered to be closely linked to mental illness and to major forms of narcotic addiction.

For illustration, we will briefly describe the results of the quantitative analysis of somatostatin, or prosomatostatin-derived peptide (PSDP) containing neurons in monkey neocortex.

The distribution of the prosomatostatin-derived peptides (PSDP), somatostatin-28 and somatostatin-28<sub>1-21</sub> in the cynomolgus monkey (*Macaca fascicularis*) neocortex was characterized in quantitative immunohistochemical studies of 3 visual areas: (V1, primary visual cortex; V2 the adjacent visual association area; and AIT, a visual association area in the anterior inferior temporal cortex), 2 auditory areas: (A1, primary auditory cortex; and T1, an adjacent auditory association area), and anterior cingulate cortex (Area 24). The results of similar quantitative analysis in 3 homologous areas in rat neocortex (primary visual, primary auditory, and anterior cingulate) are also presented. Previously, we quantified the distribution of PSDP-

immunoreactive cells in five additional regions of prefrontal cortex (Lewis et al. J. Comp. Neur. 248:1-18, 1986).

Cynomolgus monkeys were deeply anesthetized with ketamine hydrochloride (25mg/kg, i.m.) and pentobarbital sodium (10 mg/kg i.p.). Each monkey was intubated with a 3.0 or 3.5 pediatric size endotracheal tube and mechanically ventilated with 100% oxygen. After the chest was opened and the heart exposed, 1.0-2.0 ml of 1% aqueous sodium nitrite was injected into the left ventricle. In both rats and monkeys, the descending aorta was clamped and the animals perfused transcardially with cold 1% paraformaldehyde in PBS for 30-60 seconds followed by perfusion with 4% paraformaldehyde in PBS for 8-10 minutes. The brains were removed, cut into 3- to 5mm-thick blocks, and placed in cold 4% paraformaldehyde in PBS for 6-8 hours. These blocks were washed in a series of cold, graded sucrose solutions, then frozen and coronally sectioned in a cryostat. Adjacent 40  $\mu$ m sections were collected for immunohistochemical studies and Nissl staining.

For immunohistochemical studies, sections were incubated overnight at 4°C with antisera S320 or S309 diluted 1:4000 and 1:3000, respectively, in PBS containing 0.3% Triton X-100 and 0.5 mg/ml of BSA. The sections were then processed by the avidin-biotin method (Hsu et al., 1981) using Vectastin ABC kits (Vector Laboratories, Burlingame, CA) and diaminobenzidine (DAB). They were then mounted on gelatin coated slides and air-dried. In some sections, the DAB reaction product was intensified by serial immersions in 0.005% osmium tetroxide (6-7 min), 0.5% thiocarbohydrazide (10 min), and 0.005% osmium tetroxide (2 min). Other sections were counterstained with either cresyl violet or with a modification of the Giemsa method. Cytoarchitectonic areas were identified according to published criteria. Regional and laminar distribution patterns of PSDP immunoreactivity were determined by comparing immunohistochemical sections with adjacent Nissl-stained sections and by the examination of counterstained sections.

Sections reacted with S309 and Giemsa counterstained were used for the quantitative studies. The sections were examined at a magnification of 250x with a Zeiss inverted microscope. The microscope was equipped with Burleigh optical position sensors to detect stage position. The microscopist made the determination whether a neuron was immunoreactive, centered the ocular reticle on the neuron, and the x-y position of the cell was entered into a DEC LSI 11.73 microcomputer via the sensors. Laminar boundaries were entered in a similar fashion at a magnification of 63x.

Two-dimensional maps were constructed and the number of immunoreactive neurons per cortical lamina were tabulated by the computer system. A majority of the soma of S309-

immunoreactive neurons were incompletely stained. In some cases, immunoreactivity had to be contained within a Giemsa counterstained soma to be considered a PSDP-labeled neuron. Quantitization of labeled cells in monkey cortical areas were made along a 500 $\mu$ m wide cortical traverse from the pia to a depth of 400  $\mu$ m into the white matter. Cells were counted from 5 sections for each cortical area. The results were expressed as the number of labeled cells along a 1.0 mm cortical traverse. Cells were counted in monkey cortical Areas A1, T1, V1, V2, and AIT. Cell counts for anterior cingulate (Area 24) were adapted from our study of the prefrontal cortex (Lewis et al., 1986).

Primate cortical areas differed significantly in both density and laminar distribution of PSDP-immunoreactive profiles (See Fig.3.0-1 and Table 3.0-1). Area 24, the most densely labeled area, had nearly 6 times as many PSDP-immunoreactive neurons as V1. Both auditory areas contained approximately two-thirds the number of PSDP-immunoreactive neurons found in Area 24; however, both had nearly 4 times as many immunoreactive neurons as V1. The 3 visual areas showed incremental increases in the number of PSDP-immunoreactive neurons: V2 contained nearly twice, and AIT nearly 3 times the number of immunoreactive neurons present in V1. Both the supra- and infragranular layers were densely labeled in Area 24 and Area T1; however, in A1, V2, and AIT the infragranular layers were relatively sparsely labeled. In contrast to the regional heterogeneity found in the primate neocortex, the distribution of immunoreactive neurons was quite uniform across the most cortical areas.

In contrast, cortical areas in the primate obtained from a previous study contained substantially fewer immunoreactive neurons than most of the monkey cortical areas, and a majority of these immunoreactive neurons were located in the infragranular layers (See Fig.3.0-2).

These findings suggest that the regional specialization of primate neocortex involves the selective distribution of PSDP-immunoreactive neurons. They also suggest that chemically specified intrinsic organization of neocortex is not likely to be uniform across species or across cortical areas in the primate. The distinctive regional distribution patterns of PSDP-immunoreactive profiles appear to parallel that of the long corticocortical projections (contralateral and distant ipsilateral projections), suggesting an association between these presumed inhibitory interneurons and this important extrinsic system.

These data sets provide quantitative region by region and layer by region statistically meaningful comparisons of the density of this cell type, and of the relative density of this sub-cortical afferent projection. The cell data and terminal field projection data, when accumulated for human

brain, will provide a normative statistic for these regions that can be the basis for neuropathological comparisons and for functional hypotheses of the regulatory and information processing roles of these systems.

The comprehensive analyses of these two markers were accomplished over 9 months by 2 post-residency physicians not previously trained in microscopy, and would clearly have been an impossible task without the computer assisted methods developed under this contract for improved speed and accuracy of microscopic image analysis. The data analyses were obtained with equipment already on hand due to the delayed delivery of the equipment provided by this subcontract. Once the Merzhauser stage has actually been appropriately modified by its manufacturer and incorporated into the SCRF analysis system, it will be possible to transfer the 2-dimensional data and their statistical representation onto a coordinate system that can be applied to the wire frame monkey brain atlas being developed by the UCSD lab.

These kinds of microscopy analysis subroutines have obvious applications to a variety of human neuropathologies, viz: quantitative analysis of the regional and cellular distribution of amyloid plaques and intraneuronal tangles that mark the brains of patients dying with a diagnosis of Alzheimer's Disease. We have employed both classical plaque and tangle stains (Thioflavine S, or Congo Red, or Bielschowsky stains) or utilizing in situ hybridization of an mRNA probe to the amyloid beta protein gene recently shown to represent the primary genetic defect in the familial form of this disorder. Our experience makes it clear that these kinds of quantitative data can be incorporated in 3-D brain maps.

### 3.3 Organizational Wireframe Map of Human Thalamus Based on Slides from Yakovlev Collection (AFIP)

A map of the structural organization of the human thalamus was created from brain SP-18 of the Yakovlev Collection. The cytoarchitectural units composing the thalamus were outlined by interactive digital means at (0.7 mm). These were displayed as page maps using graphic plotting. Evans and Sutherland vector graphics system was used to digitize the outlines of the thalamus together with outlines of each nucleus and their subdivisions. Registration of the serial sections was achieved in reference to internal stereotaxic markers which were introduced before the brains were prepared for slicing. Thus, the protocol for achieving registration differs from that using RCI's. Nevertheless, a composite, wire frame computer image has been created to depict the thalamus and it's nuclei using conventional anatomical techniques of serial section reconstruction and alignment procedures.

The outlined nuclear regions were sampled at various magnifications as demonstrations of the cellular types and organization. These were prepared as references of each thalamic subregion by storage of images on an analogue optical disc. The optical disc is being indexed for reference to the graphic outlines of the thalamic regions and each nucleus is being labeled. The objective is to establish methods for presentation of brain maps and three-dimensional renderings of internal objects and the brain surface. These preparations served as a test bed for defining methods of data presented as described in SOW 4.0.

### 3.4 Mapping Amacrine and Ganglion Cells of the Retina

#### Development of Improved Methods for Online Quantitative Microscopy of the Retina

Description: The original Scope of Work stated that the goal was to provide quantitative information on the histochemical organization of the retina, with description of cell types, density of individual cell types, and synaptic relationships of these identified neurons. This information was to form a database on the structure function pattern of organization of the retina nerve net. Since the major goal was to establish a common database applicable to all neural circuits, the retina as chosen as a model system to develop methods for classification of cell types and quantification of their numbers. Furthermore, these data are being used to determine the limits and feasibility and utility of a database for data retrieval of information on circuits and spatial modeling. The retina, perhaps more than any other neural structure, is substantially constant in its pattern of organization in all classes of vertebrates.

The major goal is to establish a database of retina, with particular reference eventually to the human retina. Thus the selection of animal to be used was to be based on that deemed most appropriate for the overall goals of the project, with particular regard to the similarity to the human retina. These similarities included a) mixed rod and cone retina; b) well differentiated populations of amacrine and ganglion cells, and a well differentiated inner Plexiform layer (IPL). The first phase of this project has concentrated on the development of methods for the collection of quantitative spatial information. For these purposes several methods were evaluated and developed for optimal tissue preparation, staining and quantification.

In view of these considerations, the original proposal specifically stated that all initial work was to be done in the pigeon and related species, in view of the precise and elaborate degree of development of the retina in these animals, and its close similarities to the human retina. The original proposal made no mention of the use of rats or

cats, although we may eventually extend this work to include these species (on basis of other support) in view of their widespread use in studies of central visual pathways. Both of these species pose particularly difficult constraints in view of the extremely poor degree of development and differentiation of amacrine and ganglion cells, possibly related to their nocturnal adaptation and consequently predominately rod retinae. In comparison, the human retina, as in pigeons and chicks, contains a high density of cone photoreceptors. Further evidence of the limited value of the rat and cat for initial studies of mapping of amacrine and ganglion cell density and distribution is reflected in the ratio of the outer nuclear layer (ONL) vs. the inner nuclear layer (INL). In rat and cat, the IPL is very thin and poorly differentiated and has a limited diversity of amacrine cell population, and low ganglion cell density. This reduces the prospect for characterizing the cell types on the basis of their dendritic morphology within the IPL.

The nAChR staining in the retina is limited to two populations of retinal neurons, amacrine and ganglion cells. Previous studies of vertebrate retinae have demonstrated that acetylcholine and ChAT (choline acetyltransferase) is restricted in distribution to a limited population of amacrine and displaced amacrine cells. Their density of distribution in the central retina is approximately 3,000 cells per square millimeter for each of these two groups of amacrine cells. Double labeling the chick and pigeon retinae for both ChAT and nAChR has demonstrated that there is no apparent co-localization of these two substances in any amacrine cell. This implies that the cholinergic amacrine cell does not receive any direct input from other cholinergic neurons.

nAChR is found in all displaced ganglion cells (DGCs of Dogiel), and the pattern of staining permits us to trace extensive dendritic arbors out to tertiary dendritic branches. Within the ganglion cell layer proper, the intensity of nAChR staining is highly variable. Classification of the cells on the basis of intensity will require further development of software for control of the Leitz microdensitometer.

Initial estimates of the number of nAChR positive ganglion cells indicates that only approximately 15% of cells in the ganglion cell layer are cholinceptive. These estimates are very likely to prove conservative and are mainly based upon the staining pattern observed with MAb 210, a monoclonal antibody directed against nAChR. MAb 210 appears to be highly specific, but may yield an artificially low estimate of the final number of nAChR containing neurons. Studies with MAb 270 indicates a significantly larger percentage of nAChR containing ganglion cells, but still never more than approximately 30% of all cells in the ganglion

cell layer. MAb 210 also provides precise overlap in distribution with the dendrites of CHAT containing cells within the inner plexiform layer. We plan to complete the assessment of the differential staining of MAb 210 and MAb 270 within the Fifth and Sixth Quarters.

Approach: An important stated goal of this initial period was to develop improved methods for the rapid collection of data at a microscope workstation using video digitization of weakly fluorescent preparations. This method requires the use of multiple fluorophores, simultaneous differential interference contrast optics and low light video imaging. The equipment required for these techniques is undergoing rapid change and improvement and considerable amount of time was spent evaluating the feasibility of various hardware configurations, and negotiating with manufacturers for modifications in design. Establishment of this facility was further delayed for six months while awaiting approval for the necessary purchases from the DOD contract office.

We have recently accepted delivery on the modified Leitz Orthoplan II microscope with modified fluorescent attachment to allow the use of three fluorescent cubes, video port, motorized stage and microspectrophotometer. This unit permits simultaneous fluorescence and differential interference microscopy. The video image is captured using a Phillips/Mullard image intensifier with a 1:1 relay lens and a CCD camera. This unit has a sensitivity still further to  $10E-7$ . The signal/noise ratio, linearity of geometric and luminous response are substantially better than obtainable with any previous units, including SITCAMs, ISITs, or NEWVICONs. The output of the video is directly fed into a video digitizing circuit for frame averaging and image processing. This unit allows us to image directly and record the presence of three different fluorophores marking, e.g., a retrograde marker such as fast blue, and two antigens with fluorescein and rhodamine, respectively. The localization of each of these markers is clarified by simultaneous visualization of the tissue with differential interference microscopy. The resultant image can be processed for quantification of cell types and content.

We are currently beginning development of the interface to enter the data directly into the database. In the interim we plan to utilize a version of EMMA as the database system (see report by Bloom, et. al. of the SCRF). Upon completion of further modifications of the motorized stage controller and light train shutters, the CELLMATE software will be implemented with which we can rapidly re-map the preparations at high resolution. Implementation of CELLMATE (See Section 1.1.) using a motorized stage will allow us to achieve high resolution mapping of the cells. This will provide precise information regarding the density gradient



and cell sizes of the various nAChR staining ganglion cells. At the present time, this system is already providing data not previously readily attainable.

Results: Figures 3.0-3, 4, and 5 provide initial maps of the density of distribution of three parameters of retinal ganglion cell subpopulations: a) Tyrosine hydroxylase containing displaced ganglion cells in the pigeon retina; b) Substance-P like immunoreactive containing orthotopic ganglion cells in the chick retina; and c) a density map of the chick retina demonstrating the minimal density and distribution of retinal ganglion cells containing nicotinic acetylcholine receptors.

In the course of these studies on the dopaminergic amacrine cells, we also discovered the existence of an adrenergic population of horizontal cells in the rat retina. We are presently preparing tissue to obtain quantitative information regarding the density of these cells.

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Table 3.0-1

Table 1. Relative<sup>1</sup> laminar distribution of S309-immunoreactive neurons in 6 cortical areas of the monkey

Layer	Cortical areas						Difference <sup>3</sup>
	V1	V2	AIT	AI	T1	24 <sup>2</sup>	
II	17.2 <sup>a</sup> (3.7)	19.6 <sup>a</sup> (3.5)	13.5 <sup>b</sup> (0.8)	19.2 <sup>a</sup> (2.5)	17.0 <sup>a</sup> (1.7)	2.3 <sup>c</sup> (2.4)	$F_{3,24} = 30.5, p < 0.001$
III	37.7 <sup>a</sup> (11.3)	57.2 <sup>b,c</sup> (5.8)	60.3 <sup>b,c</sup> (1.8)	62.7 <sup>b,c</sup> (3.8)	39.2 <sup>a</sup> (2.6)	52.6 <sup>b</sup> (7.6)	$F_{3,24} = 13.6, p < 0.001$
IV	6.1 <sup>a</sup> (3.6)	1.2 <sup>b</sup> (1.8)	0.5 <sup>b</sup> (1.2)	0.7 <sup>b</sup> (1.2)	0.9 <sup>b</sup> (0.6)	—	$F_{4,20} = 7.3, p < 0.001$
V	5.2 <sup>a</sup> (4.5)	10.6 <sup>b</sup> (4.7)	12.2 <sup>b</sup> (0.8)	9.1 <sup>a,b</sup> (1.7)	23.3 <sup>c</sup> (3.6)	27.5 <sup>d</sup>	$F_{3,24} = 37.6, p < 0.001$
VI	33.8 <sup>a</sup> (4.9)	11.4 <sup>b,c</sup> (2.6)	13.5 <sup>b,c</sup> (0.9)	9.3 <sup>b</sup> (2.1)	19.6 <sup>d</sup> (1.1)	17.6 <sup>d</sup> (3.2)	$F_{3,24} = 48.3, p < 0.001$
Total neurons <sup>4</sup>	55 <sup>a</sup> (16)	112 <sup>b</sup> (13)	151 <sup>c</sup> (5)	208 <sup>d</sup> (9)	212 <sup>d</sup> (15)	303 <sup>e</sup> (64)	$F_{5,24} = 46.6, p < 0.001$

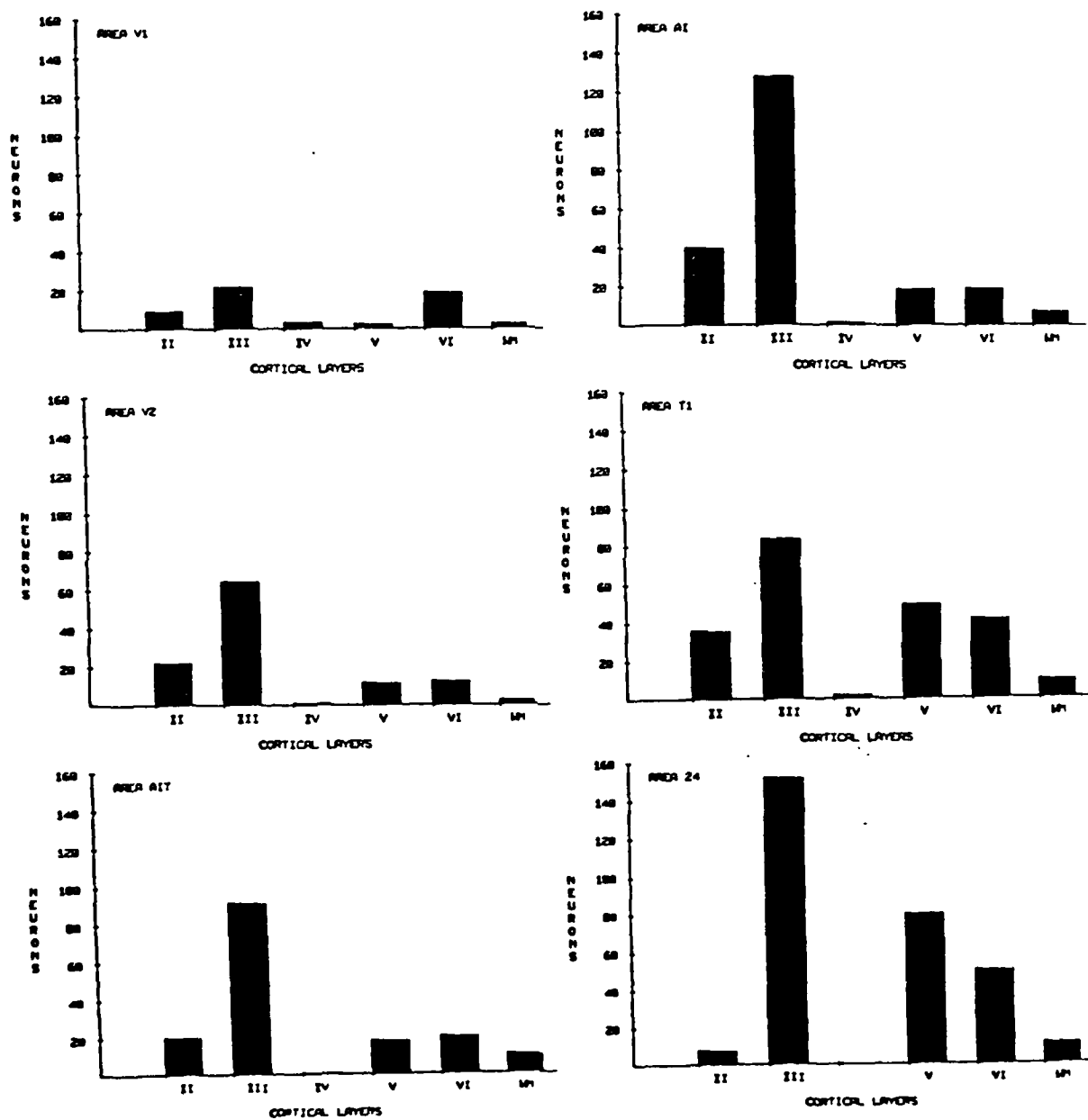


Fig. 3.0-1

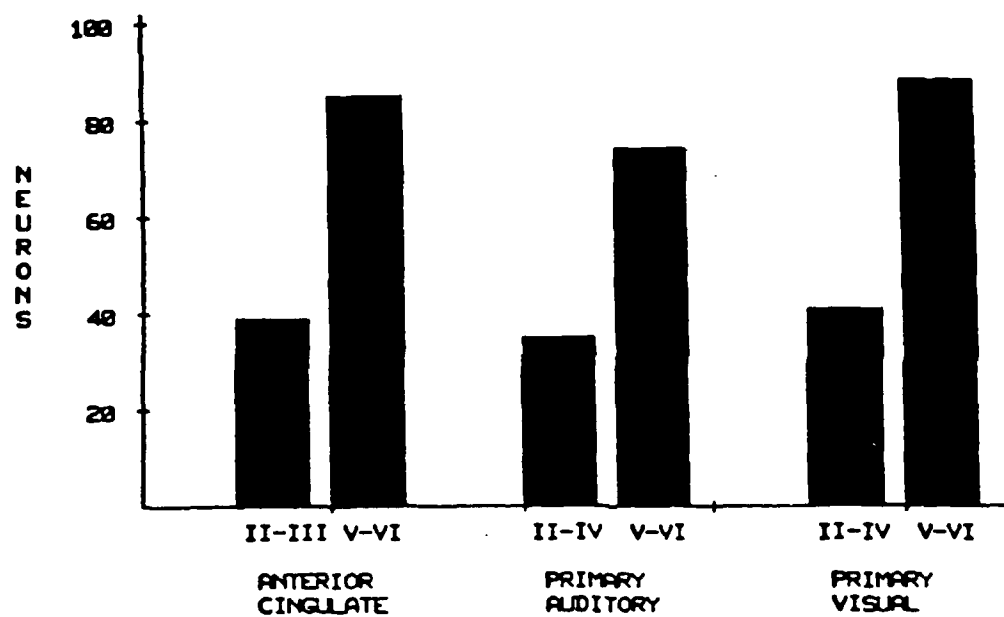


Fig. 3.0-2

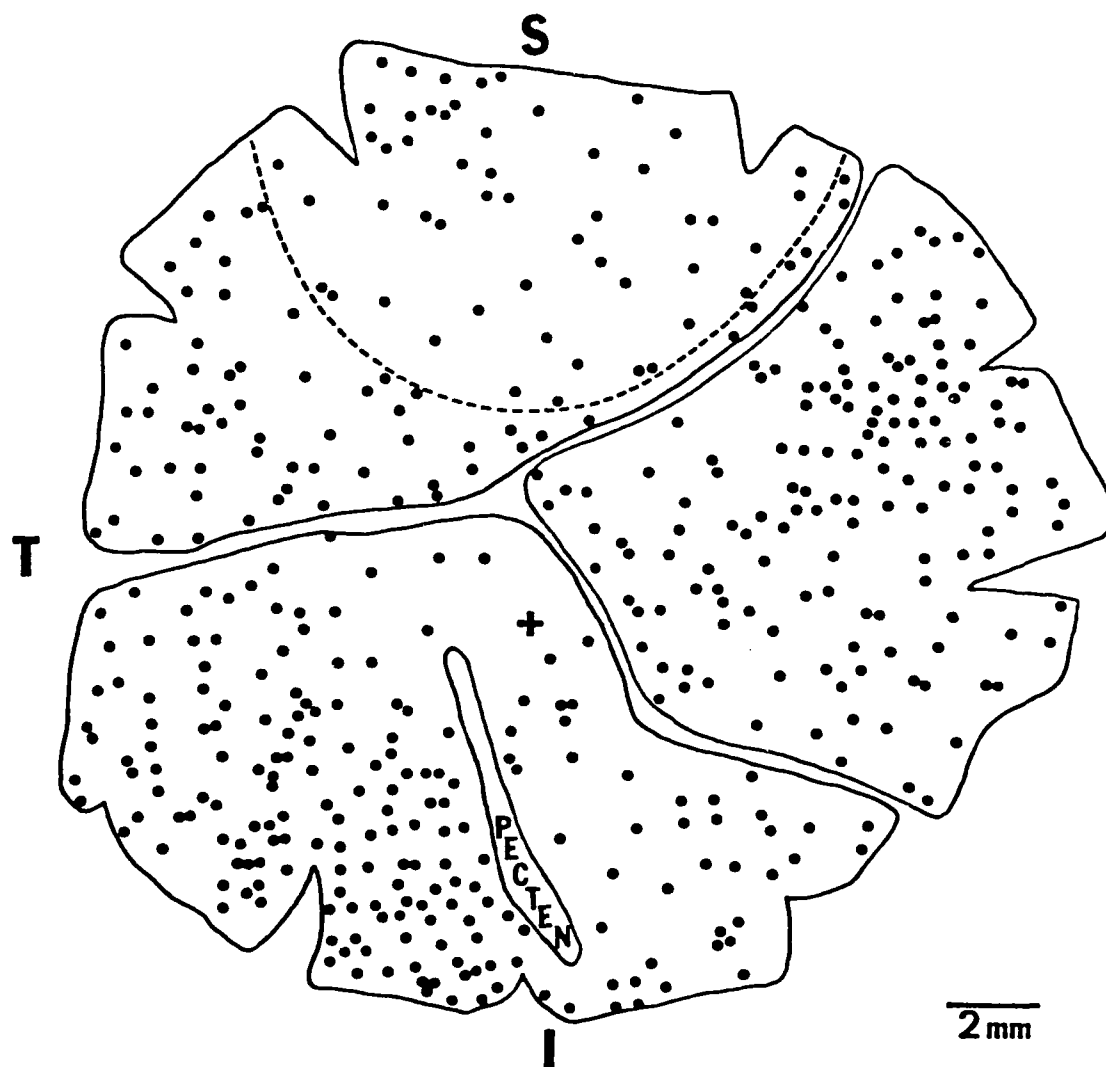


Fig. 3.0-3

Tyrosine hydroxylase positive displaced ganglion cells in pigeon retina. These cells project upon the medial terminal nucleus of the accessory optic system. Each dot = 1 cell.

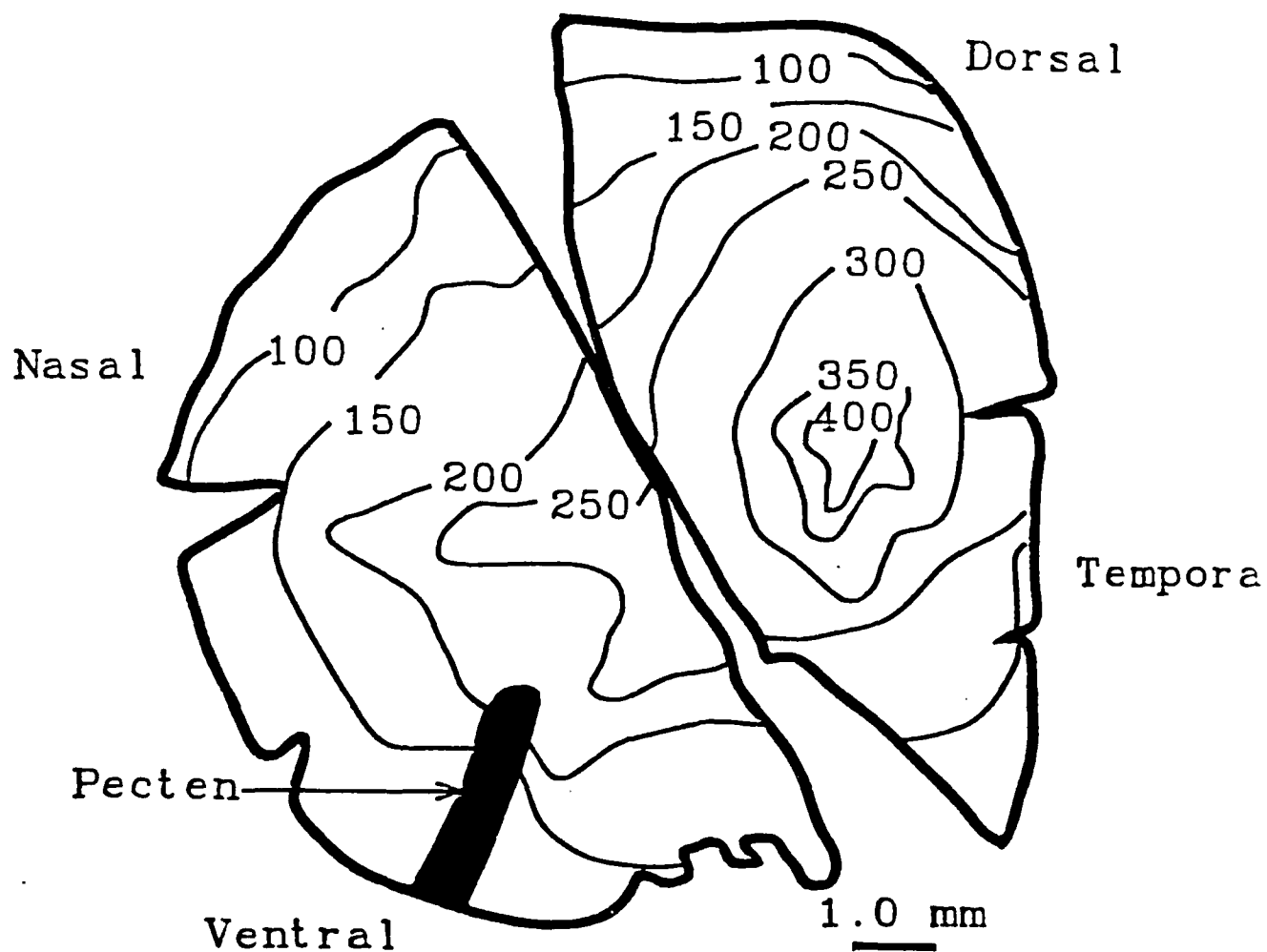


Fig. 3.0-4

Isodensity map showing the distribution of Substance P like-immunoreactive (SP-LI) retinal ganglion cells in the chick retina.

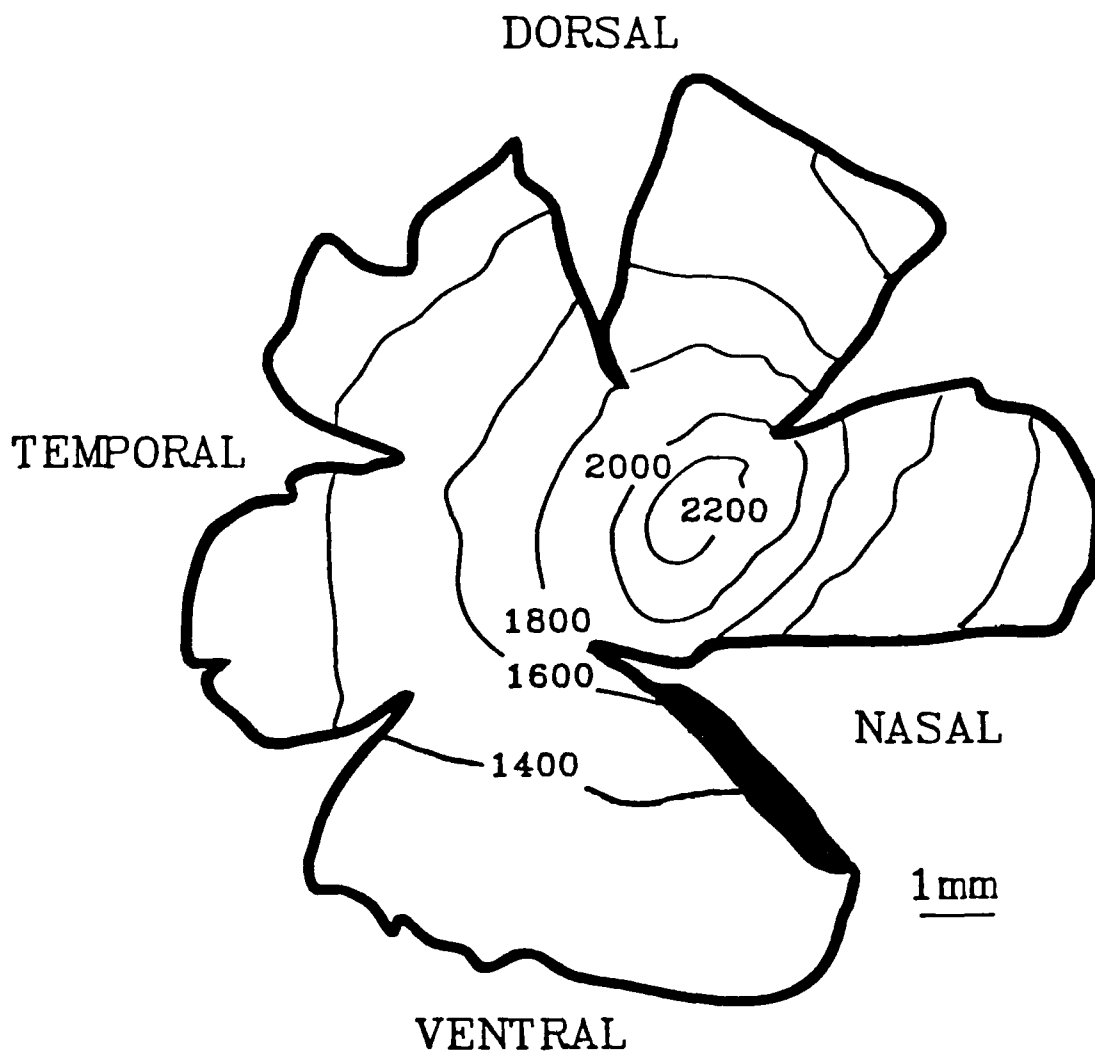


Fig. 3.0-5

Isodensity map of the distribution of nicotinic acetylcholine receptor containing retinal ganglion cells in the chick retina, using MAb 210. This map represents a minimal estimate of the density of nAChR containing retinal ganglion cell.

#### **4.0 Create Cytoarchitectural and Myeloarchitectural Reference Brain Atlases.**

**Description:** This project has two major parts. The first is the development of a human brain reference atlas of analogue images on an optical disk. These images are from celloidin stained sections of slides from the Yakovlev Collection. The second part of the study is to generate digital renderings of human brain surfaces, ventricles and surfaces generated from myelo- and cytoarchitecturally defined contours made available by mapping technology.

#### **4.1 Human Reference Atlas of Images of Various Brain Structures.**

Creation of a cytoarchitectural and myeloarchitectural reference atlas of the human brain was carried out using material from a single, serially-sectioned, celloidin embedded brain from the Yakovlev Collection at the Armed Forces Institute of Pathology (AFIP) in Washington, D.C.

Subcortical structures were mapped on photographic enlargements having a magnification of 12.5x of whole brain Nissl and myelin stained sections using a dual stage microscope that permits optical superposition of adjacent serial sections. Mapping of cells constituting the subcortical cytoarchitectural units was carried out under the microscope at 40x, 100x and 200x, and photographic documentation of each of the anatomical features of interest was provided on 35 mm color slides.

Serial tracings of the brain cytoarchitecture were created at an interval of 0.7 mm to show the location of subcortical nuclei which were then named in accordance with conventions found in the recent literature. Serial maps of brain myeloarchitecture were created at an interval of 0.7 mm using 35 um sections adjacent to those employed for the cytoarchitectural study; the principal myeloarchitectural units were identified on tracings of the maps.

Photographic documentation of the cytoarchitecture and myeloarchitecture in the form of several hundred 35 mm slides was reconfigured first as a series of NTSC video images on 1/2 and 3/4 inch magnetic tape and later as part of a videodisc on human anatomy and pathology created at the University of Utah. These materials were indexed to the appropriate level of the atlas by numbers referenced to the original section levels, and the names of the features were displayed in association with each group of photomicrographs. A summary table of contemporary nomenclature for the human cytoarchitectural units has been created; it will be supplemented by a list of equivalent terms for structures in the rat brain, based on current literature.



A contour atlas has been traced that depicts the outlines of the mapped features at all levels through the brain together with designations of each of the units by an identification number. These digital representations were generated by interactive tracing of the boundaries of structures from enlarged photographs of the brain sections. Each structure was given a corresponding scientific name, both of which, are also listed in a directory. These boundaries are now ready for wire frame representation and 3-D rendering as indicated in SOW 4.2.3 and 4.2.4.

#### **4.2. Digital Renderings of Myelo- and Cytoarchitectural Features Defined in Stained Celloidin Sections.**

Digital renderings of all cytoarchitectural features constituting subcortical gray matter of the human brain have been created using an Evans and Sutherland Picture System 2 by manually digitizing the outlines of the units at each of the mapped levels. The 3-dimensional shape of the nuclei were then reconstructed in a series of wire frame images aligned with respect to internal reference marks. The latter had been introduced into the brain after death by means of a stereotaxic device. Work is now in progress to render each of the units as a surfaced, shaded 3-dimensional object by means of more advanced computer graphic techniques.

Problems encountered: Currently, a robust and effective software approach to three dimensional reconstruction has not been provided by vendors. The rendering approaches and the wire frame display software is adequate. However, software for generating triangulation, polygons and surface depictions has not been satisfactorily designed.

#### **General Problems Encountered:**

##### **1. Receiving Equipment and Software.**

Task accomplishment has been made unexpectedly slow and difficult because of unforeseen delays and difficulties. These were due to: Vendor delays in supplying equipment; Software unavailability from vendors; Delayed vendor software development which software was expected as required for brain mapping. Protocol development for generating exemplary preparations has taken about 6-7 months longer than anticipated. Great extra effort was required, due to the inadequacies of vendor software and hardware. Although specifications were well defined, in actuality the hardware and software did not function at the level that was required. This may have been largely because the systems we required are on the cutting edge of commercially available technology.

## **2. Bottlenecks Involving the Workstation.**

When data are being acquired, programmers cannot work and vice versa, a problem solved by data acquisition being relegated to nights and weekends, permitting programmers full access during working time. A secret bug somewhere in software allows the MegaVision to crash the whole system.

## **3. Limitations of the Systems Acquired.**

How fast does the MegaVision acquire images from the block-face? Accumulation and averaging (128 : 128) takes about 43 seconds; storage takes 16 seconds. These times vary according to the concurrent use of the computer so that these times are minima. Using the ABM Slicer there is one step for accumulate, divide and store with file name, which takes 55 seconds.

For storing an image: a 1 MB image takes about 15 sec. to be put onto disk, and about an equal time to recover it from disk to MegaVision. Movement of a 1 MB image should take only about one second. To tar tapes: Directories on the disk vary from 70 to 85 megabytes; each directory takes about 4.5 hours to dump onto a tar tape. One rat brain, sectioned at 50  $\mu$ m, fills 8 directories, which takes 36 hours to dump.

Limitations of speed of operations in commercially available equipment has revealed bottlenecks where specialized equipment will need to be developed for improved brain mapping. These areas include image acquisition, frame storage and retrieval, and three dimensional image reconstruction. Higher resolution images (i.e. 12 minimal, better 16 bits per pixel, with 2K to 4k squared images) demand novel approaches to manipulate with the 4-32 megabyte images. Methods of image compression and rapid storage are needed to manage the data. Moving of 1024 squared frame images at near real time will be necessary. Solutions to these problems are being developed by some vendors. Recent advances in display generation for presentation of data suggest that 3-D renditions of structures can be displayed with hardware devices. Both General Electric and Evans and Sutherland in combination with DEC have now achieved this potential; machines will be available within the year as systems that have resolutions and speeds that are within the time ball park necessary to interact reasonably efficiently with brain reconstructions. One of the gaps is in machinery or very competent software that will provide large data polygon surface renderings to all kinds of biological shapes. When such surface renderings can be provided, there are machines that can already provide beautiful, dynamic displays rapidly.

## Discussion and Projections of Future Work

Some of the major technical obstructions to registration control imaging have been overcome during the year. These included creating an interface between the LKB microtome and the RCI digital camera, preparing the specimens in such a way as to allow reasonable white/gray matter differentiation, and determination and implementation of a reasonable sequence of MegaVision processes for image acquisition. We have succeeded in capturing trial RCI's from rat whole heads and brains sectioned in the coronal plane, and we are creating wire-frame "atlases" [SOW 1.0].

We have worked extensively on the difficult problem of making the macro-micro transition without a loss of either registration or cytoarchitectural information. Our results with thin-section collection are encouraging, and with the implementation of ex situ PEG sectioning (see below) we are confident that the material obtained for TSI mapping will be acceptable [SOW 1.3].

Indeed, possibly the most important result of the first years' work on data acquisition has been in designing a method by which the in vivo gross morphometry of the brain, as well as the interrelationships between the brain, meninges, and skull can be maintained (or at least reconstructed) without the necessity of in situ sectioning. Although the LKB giant cryomicrotome is capable of cutting through skull (and even teeth) the results of cutting in the skull are poor, yielding sections of low quality and causing rapid knife edge deterioration. Thus, in order to progress from the simple "cryoplaning" adequate for RCI's, to the ability to collect serial tissue sections of sufficient quality for microscopic TSI work we are implementing a radical departure from conventional sectioning procedures.

### Polyethelene Glycol Embedding for ex situ Sectioning: [SOW 1.2]

Polyethelene glycol (PEG) is used extensively as a water-soluble embedding medium (Ellisman and Porter, 1980). This substance can be infiltrated into blocks of tissue by sequentially incubating them in solutions made with increasing molecular weight (MW) PEG. When the specimen is cooled from the incubation temperature (usually 40 to 60 °C) the PEG solidifies, and can be cut in a fashion similar to paraffin sectioning.

We plan to employ PEG embedding for human RCI/TSI microtomy in the following manner: After staining with vital dyes and fixation with aldehydes (or possibly osmium tetroxide) we will warm the specimen slowly by perfusing with buffer. The final temperature will be determined by the MW of the PEG employed...perhaps as high as 50 °C for 10,000

MW. When the head has reached the target temperature, we will begin arterial perfusion with ascending MW's of the polymer. This step will evenly distribute the embedding media throughout the brain. We plan to follow this by filling the subdural space with liquified high MW PEG. This will be accomplished by implanting catheters through the orbital roof, the temporal bones, the roof of the mouth and around the base of the occipital pole above the tentorium. If necessary, we will also insert catheters into the ventricles and fill them directly with high MW PEG. One of the pairs of catheters will be connected through a peristaltic pump to a PEG source, the other will operate in withdrawal mode. By positioning the pair of catheters on opposite sides of the head we can monitor the movement of PEG as it fills the subdural space by observing it's arrival at the efflux catheter.

When the subdural space is uniformly filled, we plan slowly to cool the specimen to approximately 4 °C. At this temperature we will perform a second series of MRI/CT scans to evaluate the influence of PEG on gross morphometry. If these images indicate that this procedure is morphometrically innocuous, we will carefully dissect away the skull and dura from the PEG "casting", leaving an orbito-meatal strip of skull and dura intact as a band in support of the brain. This "ring" of bone and dura will be used to align the brain stereotactically within the microtome. We will build up a mound of PEG to affix and embed the specimen on the level of the stage, when we reach the bone strip, the bone and dura will be removed, and the embedding completed. In this fashion we hope to maintain registration control from the original MRI/CT scans through the digital RCI's obtained from the blockface.

The quality of sections that can be obtained for TSI analysis by this method should greatly exceed those obtainable through cryomicrotomy. In addition, even though this preparation time is likely to prove long and tedious, the subsequent improvement in speed of sectioning should more than compensate.

In respect to RCI and TSI acquisition and three dimensional wireframe image reconstruction, we are ready to "put it all together," and interface all of the interdependent techniques. The global protocol consists of MRI/CT virtual RCI's, specimen preparation for ex situ sectioning, microtomic RCI's. Serial TSI section collection and mounting will be the next major hurdle. Concurrently with solving this problem, we will focus on the final concern relating to accurate data acquisition. This is to map MicroTSI by "strip mapping" and mosaicing, using the computer assisted TSI microscope. registration-controlled synthetic montages [SOW 1.4, 1.4.1, 1.4.2 and 2.1].

### References

Ellisman, M.H. and Porter, K.R. (1980): The microtubular structure of the axoplasmic matrix: visualization of cross-linking structures and their distribution. J. Cell Biol. 87:464-479.

## Appendix - Figure 1

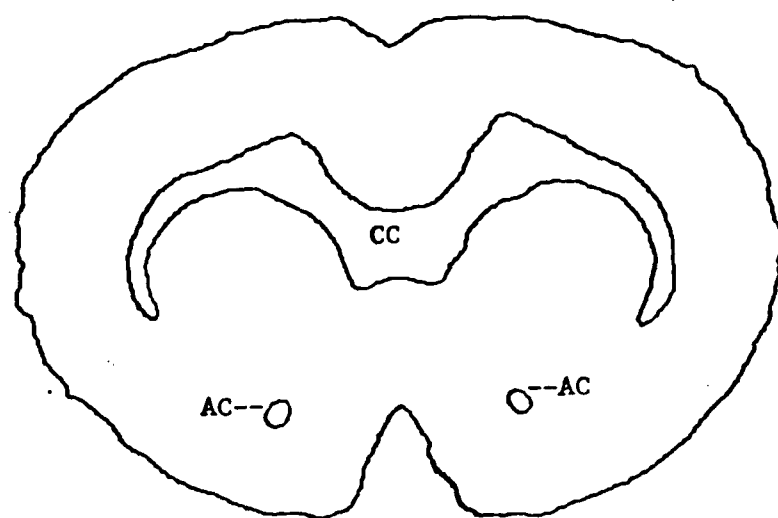
Figure 1 shows the gray-scale blockface image of a rat brain (a) and its contoured outlines (b). The rat was stained with methylene blue during perfusion. The brain was embedded in black albumin/gelatin and coronally sectioned in the cryomicrotome at 50  $\mu$ m intervals. Image (a) was captured with a MegaVision video camera and filed as a 1024x1024 pixel array with 8-bit depth. The half-tone was produced by the laser printer. This is not as good quality as the image viewed by the operator on the MegaVision monitor while sectioning and acquiring images. Differentiation between gray and white matter in the blockface was established by the camera. Image (b) shows the result of the ABM Mapper Program applied to image (a). The same operator-selected threshold was applied to all registration control images from that specimen. The contour points are interpreted by a sixel program to produce the laser printout (b). The resulting anatomical contours include an outline of the brain, the corpus callosum (CC), and the anterior commissure (AC).

Appendix - Figure 1

(a.)



(b.)



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